BASELINE TETRACYCLINE AND TETRACYCLINE RESISTANCE LEVELS IN PERENNIAL, WADEABLE STREAMS OF KANSAS AND NEBRASKA

by

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IN PERENNIAL, WADEABLE STREAMS
OF KANSAS AND NEBRASKA

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Abstract

Antibiotics and antibiotic resistance are emerging contaminants. Tetracyclines are common antibiotics with a well-known mode of action and multiple resistance determinants. Water column samples were collected from 22 streams in Kansas and Nebraska in conjunction with a USEPA probability-based study of perennial, wadeable streams. Tetracyclines were analyzed by enzyme-linked immunosorbent assay (ELISA), while polymerase chain reactions (PCR) were used to enumerate 16S-rRNA, *tetW*, *tetQ*, and *tetO*. *TetW*, *tetQ*, and *tetO* were highly correlated ($r^2 > 0.80$, $p < 0.001$) in all streams. Observed values of genes and tetracyclines were consistent with moderately impacted areas from recent targeted studies, and were generally no different among states, reference conditions, and ecoregions. However, *tetW* levels were significantly lower in Kansas than Nebraska. Based on probability, approximately 20% of Kansas and Nebraska streams are predicted to have observable levels of tetracyclines, *tetW*, *tetQ*, and *tetO*. Findings suggest an ambient reservoir of tetracycline resistance genes and favorable conditions for resistance selection.

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Introduction

Antibiotics are a powerful tool, used both for treatment and prevention of infection. However, widespread use and misuse of antibiotics can lead to development of antibiotic resistance in bacteria and other microorganisms. This resistance is genetically based and potentially inheritable. With the discovery of horizontal gene transfer, the potential for development of widespread antibiotic resistance, including resistance in pathogens, has become more apparent. Antibiotic resistance in both pathogens and other microbiota could pose significant threats to public health, public and private property, and biological communities.

This study was undertaken to provide an initial estimate of the magnitude and extent of ambient antibiotics and antibiotic resistance gene levels in streams of the Central Plains.

Current State of Knowledge

First discovered in the 1940's, tetracyclines are a class of molecules characterized by a linear, tetracyclic nucleus with a host of attached functional groups responsible for antimicrobial properties (Chopra and Roberts 2001; Kulshrestha et al. 2004). Three of the most common tetracycline variants are tetracycline (TC), oxytetracycline (OTC), and chlortetracycline (CTC), which differ by substitutions of the R5 or R7 functional groups (Figure 1). Tetracyclines are moderately persistent, non-volatile, hydrophobic compounds, capable of forming chelating complexes with divalent metals (often Mg2+) under low pH or reduced redox conditions (Chopra and

Roberts 2001; Halling-Sorensen et al. 2002; Kulshrestha et al. 2004; Meyers and Smith 1962). Chelation makes them soluble and potentially mobile in both aqueous and lipid solutions (Aga et al. 2005; Doi and Stoskopf 2000). Sorption to dissolved organic matter is also a mode of increased mobility (Halling-Sorensen et al. 2002; Kulshrestha et al. 2004). Despite this mobility, however, tetracyclines in soil are generally confined to the first 30 cm, with the exception of the few millimeters at the surface where photodegradation is still possible (Hamscher et al. 2002). Oxytetracycline, a representative tetracycline, has a molecular weight of 496.9, $pKa_1 = 3.22 \pm 0.30$, $pKa_2 = 7.46 \pm 0.03$, and $pKa_3 = 8.94 \pm 0.30$ (Qiang and Adams 2004). Oxytetracycline also has a K_{OW} of 0.11 – 0.34 and is very soluble at pH 1, 4, 7, 8, and 10.

Tetracyclines are characteristically photosensitive (Addamo et al. 2005; Halling-Sorensen et al. 2003; Oka et al. 1989), and tend to degrade more rapidly at high pH (> 8), high temperatures (> 30 °C), and in aerobic conditions in the presence of light (Addamo et al. 2005; Doi and Stoskopf 2000; Halling-Sorensen et al. 2003; Oka et al. 1989; Qiang and Adams 2004). Degradation products of tetracyclines also exhibit toxicity to microbes, though at generally reduced levels (Aga et al. 2005; Halling-Sorensen et al. 2003; Halling-Sorensen et al. 2002; Park and Levy 1988). Some degradation products, such as 5a,6-anhydrotetracycline hydrochloride (ATC), actually show increased toxicity over parent compounds, due to alternate modes of action (Halling-Sorensen et al. 2002). The typical mode of action of tetracyclines is bacteriostatic. They inhibit transcription by interference with the

attachment of aminoacyl-tRNA to the acceptor (A) site on the ribosome (Chopra and Roberts 2001; Halling-Sorensen et al. 2002). "Atypical" tetracyclines such as ATC have different modes of action (in this case, interference with membrane permeability), and are therefore capable of affecting organisms that may exhibit resistance to the parent compound. Low-level persistence of tetracyclines and their degradation products, coupled with their tendency to sorb and desorb based on environmental conditions, have been identified as a potential selective force for the development of ubiquitous gene pools conferring antibiotic resistance, particularly to drugs with similar modes of action to the tetracyclines (Aga et al. 2005; Chopra and Roberts 2001; Halling-Sorensen et al. 2002; Hamscher et al. 2002). Since tetracyclines have not been shown to be directly hazardous to humans at the concentrations in which they generally persist in the environment (ng/L to μg/L), their primary hazard to humans is the potential for fostering microbial resistance to antibiotics.

Applications for tetracycline are varied, spanning pharmacological and therapeutic usage in human and veterinary medicine, to animal growth promotion, and animal and plant infection control (Chopra and Roberts 2001; McManus et al. 2002). Chopra and Roberts (2001) estimate that 3.48×10^6 kg of tetracyclines were used for farm animal applications each year during the 1990s. Both prophylactic and therapeutic use of tetracyclines consists of repeated, relatively high dose administration, either by direct ingestion, or amendment to feed. Tetracyclines are also applied in solution as an injection or spray. Pathways for environmental

exposure to tetracyclines include direct application and deposition of both non-metabolized and partially metabolized tetracyclines in human and animal waste products.

Tetracyclines generally enter the environment in repeated, high doses, and accumulation of tetracyclines often occurs near the surface of soils in the form of persistent residues (Hamscher et al. 2002). The potential for low-level, chronic exposure of microorganisms to these residues is commonly identified as a threat for development of widespread antibiotic resistance, and the consequences of the common practice of preventative antibiotic use in livestock feed and application of the manure of those livestock as fertilizer may be long-reaching (Gujarathi et al. 2005; Hamscher et al. 2002; Ingerslev et al. 2001; Koeypudsa et al. 2005).

Microorganisms, especially bacteria, have a wide range of environmental functionality, such as metabolic plasticity and antibiotic resistance. Recent evidence has shown that many such functions are inheritable through coding by mobile genetic elements (MGE), such as plasmids, phages, transposons, and gene cassettes (Smalla and Sobecky 2002). Such MGE are known to persist in various aquatic environments at levels sufficient for significant interaction with organisms. Transfer of MGE between organisms is termed horizontal (or lateral) gene transfer, since genetic components are exchanged *horizontally* within a given generation of individuals, as opposed to *vertically* from parent to offspring. To be clear, however, it must also be recognized that horizontally transferred genes are themselves often vertically inheritable by subsequent generations. Horizontal gene transfer occurs by three

methods: conjugation (direct cell to cell exchange of plasmids), transduction (phage-mediated transfer of DNA similar to viral infection), and transformation (incorporation of free genetic material into existing cells). All three methods have been shown to occur in aquatic environments (Chopra and Roberts 2001; Smalla and Sobecky 2002), and transfer of antibiotic resistance has been linked to conjugation (Bruun et al. 2003; Roberts 2005).

Many mechanisms for antibiotic resistance have been documented. For example, in an extensive literature review, Chopra and Roberts (2001) noted mechanisms of tetracycline resistance had been identified for 39 genera of gram-negative and 23 genera of gram-positive bacteria and related genera. Several such mechanisms are known to be encoded by genes horizontally transferable via plasmid conjugation (*e.g.*, *tetB, tetO, tetQ*) (Chopra and Roberts 2001; Roberts 2005). It is also of some importance to note that many of the mechanisms conferring antibiotic resistance also convey some resistance to metals (and vice versa) (Baker-Austin et al. 2006; McArthur and Tuckfield 2000; Stepanauskas et al. 2006; Stepanauskas et al. 2005; Tuckfield and McArthur 2007). For example, the resistance determinants tetW, tetQ, and tetO all code for ribosomal protection. By shielding the ribosome from inhibitory attachment, this mechanism provides resistance to a wide range of compounds, including tetracyclines and some metals (Roberts 1996; Roberts 2005).

Given the documented potential for development of antibiotic resistance and the potentially dangerous implications of such resistance development, several studies have been undertaken to examine the problem. Using gene primers developed by

Smith et al. (2004b), Peak et al. (2007) found that the abundance of resistance genes (relative to the total gene count) was significantly higher in feedlot lagoons associated with tetracycline treatment than in those without it. As a next step, Engemann et al. (2006) performed mesocosm and laboratory studies to examine the environmental fate (half-life) of resistance genes under environmental conditions. In that series of experiments, tetracycline resistance genes were found to decay more rapidly with light exposure, and the disappearance of resistance genes was hypothesized to depend more on ecological controls (e.g. competition) than physical ones (e.g. photolysis). In a subsequent paper, Engemann et al. (2008) have recently shown evidence that tetracycline resistance genes decay at different rates from the water column (i.e., tetO < tetW < tetM < tetQ), and that these genes do migrate readily into biofilms, which suggests that the genes are not disappearing, but rather moving to different environmental compartments. A recent paper by Knapp et al. (2008) has corroborated this movement by documenting correlation between *tet* resistance genes and gene patterns in two transposons (Tn 916 and Tn1545), which suggests horizontal transfer is occurring. Knapp et al. (2008) also observed increases in both the relative abundance of resistance genes to 16S-rRNA genes and in the selection rate of resistance genes at low levels (20 ug/L) of oxytetracycline. Based on the combined evidence of these experiments, increases in the relative abundance of antibiotic resistance genes do seem to be occurring in the environment.

Similar targeted studies by Pei et al. (Auerbach et al. 2007; Mackie et al. 2006; Patterson et al. 2007; Pei et al. 2006; Pruden et al. 2006) have all shown

evidence of antibiotic resistance gene presence in the environment. However, very few data are available on the ambient levels of tetracycline resistance genes. Tetracycline is a naturally occurring compound, which suggests some level of natural antibiotic resistance is likely present in the environment. Moreover, since antibiotic amendment is widespread and anthropogenically produced antibiotics have been shown to be present at low levels in the environment, it is also likely that some background level of anthropogenically induced antibiotic resistance may also already exist in the environment, though Lau et al. (2003) have suggested this background may be naturally occurring, with no recent horizontal transfer from antimicrobial preparations to environmental genes. At the least, there is evidence for the potential growth of new antibiotic resistance gene reservoirs via horizontal gene transfer from livestock waste lagoons to biofilms (Engemann et al. 2008) and groundwater (Chee-Sanford et al. 2001; Koike et al. 2007; Mackie et al. 2006).

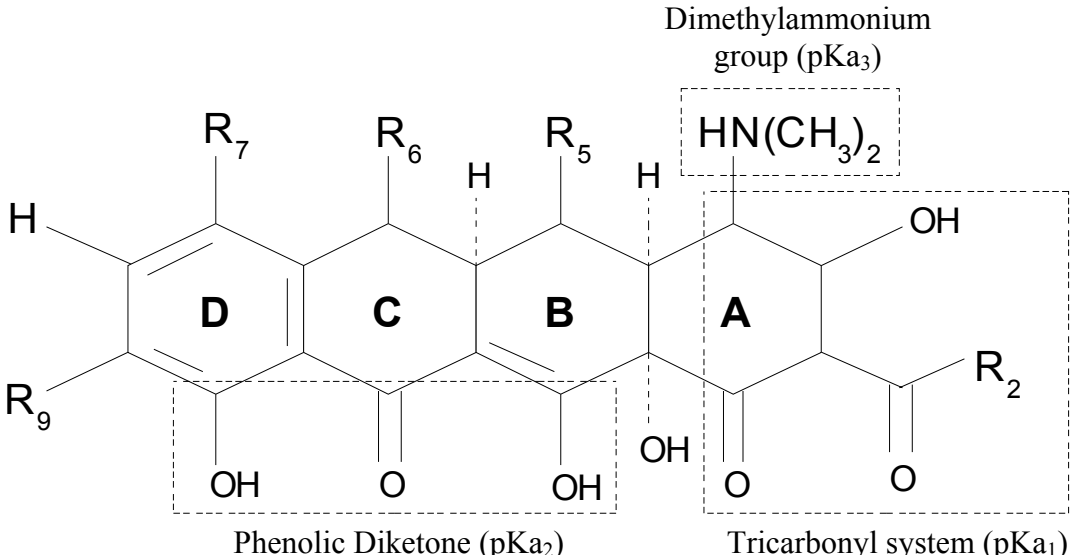

Figure 1. Structural diagram of the tetracyclines.
Substitution of the various R groups yields different compounds. For example, chlortetracycline has R7 = Cl and oxytetracycline has R5 = OH. The dashed boxes indicate the functional groups associated with the three pKa values of the compounds.

Q0: Project Description

This project began with a two previously unanswered questions about tetracyclines and tetracycline resistance genes. First, what are the ambient or baseline levels of tetracyclines and tetracycline resistance genes in the environment? Are there observable patterns of abundance and distribution for either tetracyclines or their resistance genes, and can the spatial extents of these patterns be predicted for areas that are not sampled? Second, are levels of tetracyclines and tetracycline resistance genes levels related in the environment? For example, do similar resistance genes have similar or different baseline levels or responses in the environment?

To answer these questions, perennial, wadeable streams of Kansas and Nebraska were selected as the environmental system for study. The reasoning behind this selection is fourfold. First, anthropogenic impairment of both surface waters (Mudryk 2002; Yang and Carlson 2003) and ground water (Chee-Sanford et al. 2001; Koike et al. 2007; Mackie et al. 2006) by both tetracyclines and tetracycline resistance has been shown. Second, there is widespread use of tetracyclines in Kansas and Nebraska, especially in confined animal feeding operations (CAFOs). Third, both CAFOs (Figure 2) and wastewater treatment plants (Figure 3) are widespread in both Kansas and Nebraska, suggesting widespread potential for effluent-mediated contamination of surface streams. Finally, significant research was occurring or scheduled to occur at the University of Kansas (KU) concurrently with this study, including both novel primer development for resistance gene identification and enumeration and a larger-scale, nationwide survey of perennial, wadeable streams. As indicators of tetracycline resistance, the ribosomal protection genes tetW, tetQ, and tetO were selected for study. These genes were chosen for three primary reasons: their known presence in samples from livestock waste, human waste, and environmental samples; their potential for both vertical and horizontal gene transfer by known mechanisms; and concurrent study at KU to develop novel primers for these genes.

Therefore, to assess ambient levels of tetracyclines, tetracycline resistance genes, and their potential correlates in wadeable (1^{st} to 5^{th} order (Strahler 1957)) streams of Kansas and Nebraska, stream sites were chosen concurrently with the

National Wadeable Streams Assessment (WSA) (USEPA 2006). WSA sites were selected at random from a larger population of streams using a Generalized Random Tessellation Stratified Design (GRTS), which was developed in previous USEPA studies (Stevens Jr. and Olsen 2003). This type of probability-based sampling allows for statistically quantifiable extrapolation of the study results to a larger population than that sampled and is particularly valuable for assessing extensive resources, while limiting costs. A more detailed explanation of the sampling design is provided later (see the Predicted Values and Spatial Extents section of this manuscript). Seventeen sites in Kansas and Nebraska were selected, and five additional sites were selected as "Reference" sites, or sites with minimal anthropogenic impact and high biological quality (Figure 4). Reference condition sites were selected based on a weight of evidence or "best professional judgment" approach by officials from the Kansas Department of Health and Environment (KDHE) (3 sites) and the Nebraska Department of Environmental Quality (NDEQ) (2 sites) for their respective states. The resulting 22 sites span seven Omernik (1987) Level III ecoregions (Figure 6), and fourteen US Geological Survey (USGS) hydrologic unit codes (HUCs) (Figure 7). Watershed size, land use/land cover characteristics, and physical – chemical parameters vary from site to site (see Appendix A – Selected Watershed and Site Characteristics).

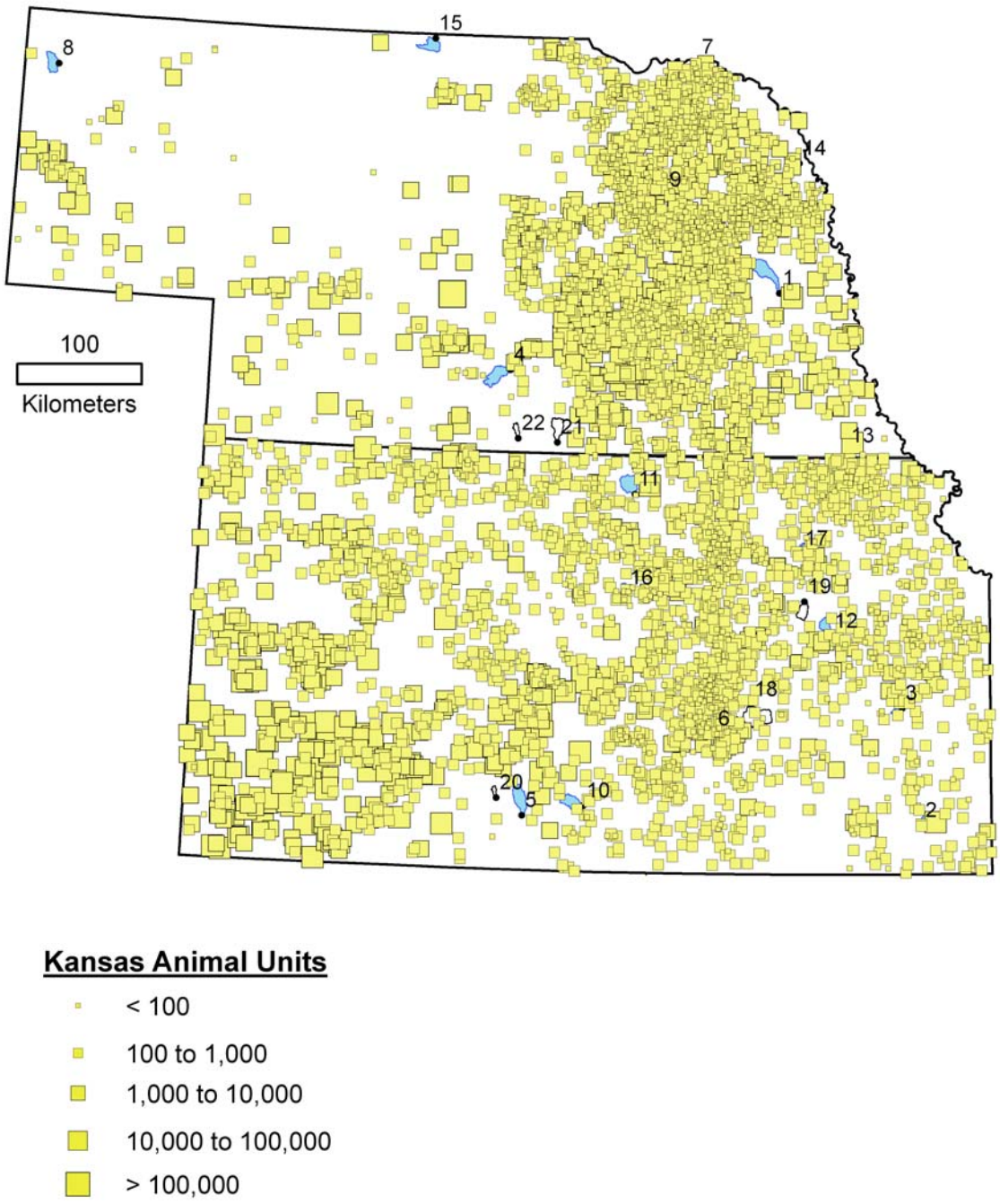

Figure 2. Spatial distribution of permitted confined animal feeding operations (CAFOs) in Kansas and Nebraska.
Relative box sizes indicate magnitude of operation in terms of standard Kansas Animal Units (i.e., the amount of waste produced by a 1,000 lb cow in one day) (KDHE 2009). Delineated watersheds for project study sites and site numbers are also indicated.

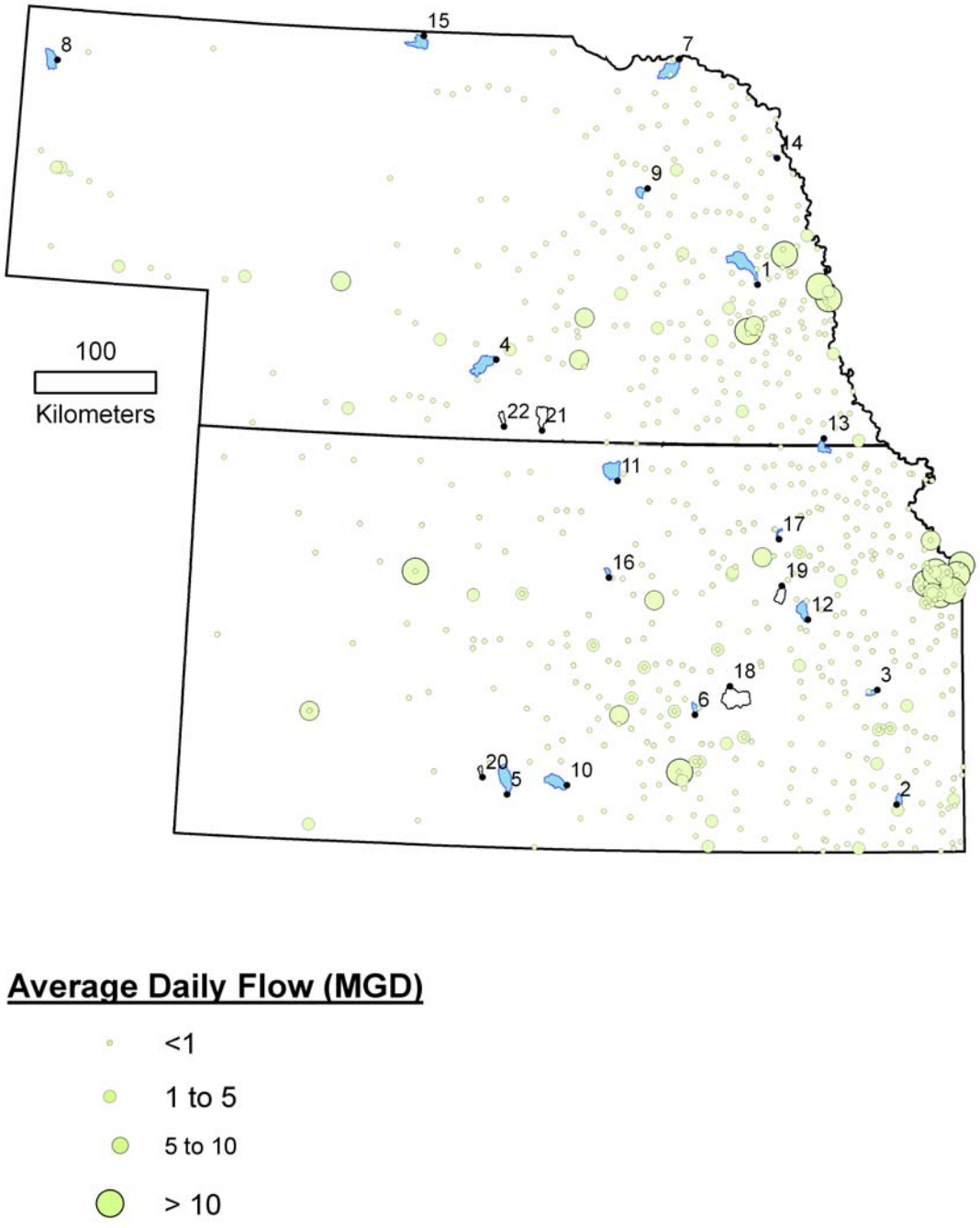

Figure 3. Spatial distribution of permitted municipal wastewater treatment facilities in Kansas and Nebraska.
Relative size of circles indicates magnitude of flow. Black points indicate project study sites with site numbers. Delineated watersheds are indicated to scale by closed irregular polygons. Filled (blue) polygons indicate probability sites, while empty (white) polygons indicate best professional judgment reference sites.

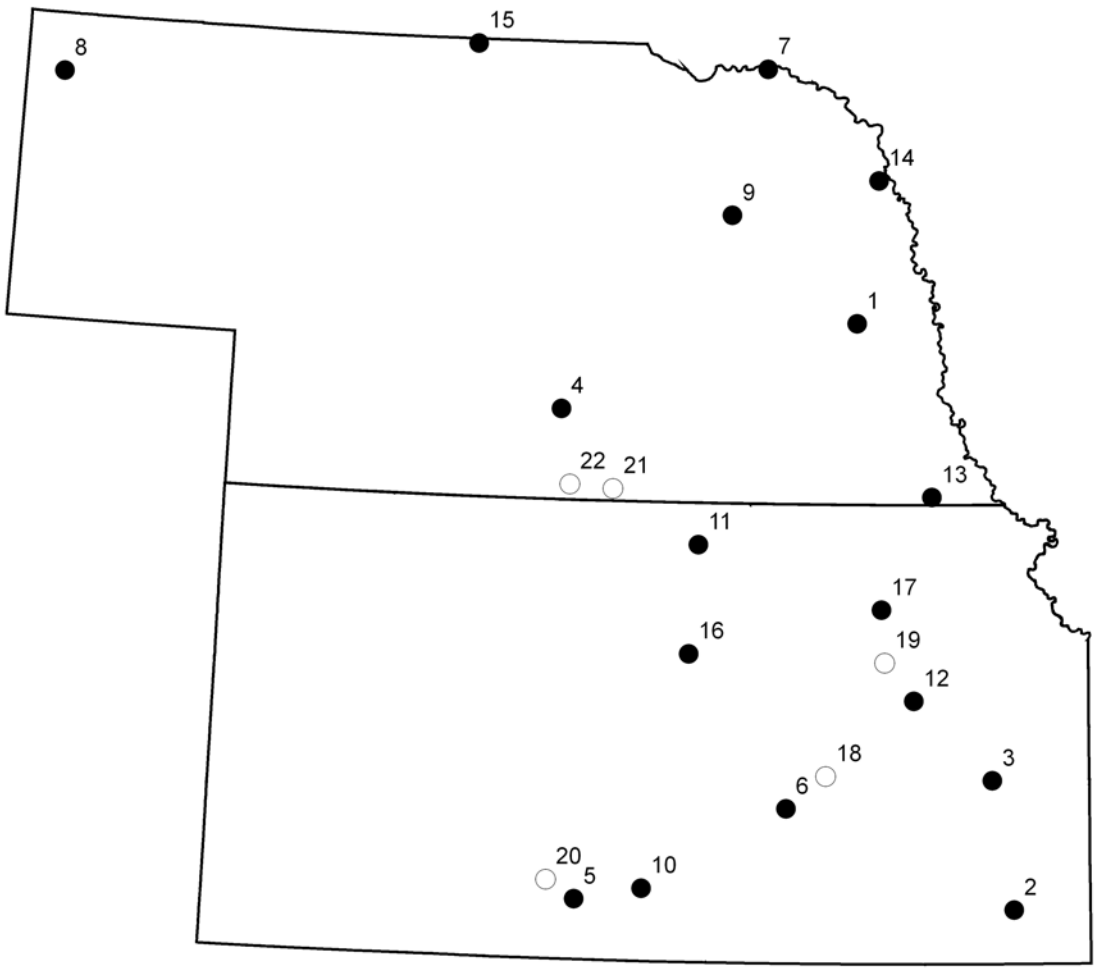

Figure 4. Location of sampling sites.
Open circles indicate reference condition sites selected by best professional judgment, while filled circles indicate probability-selected, non-reference sites.

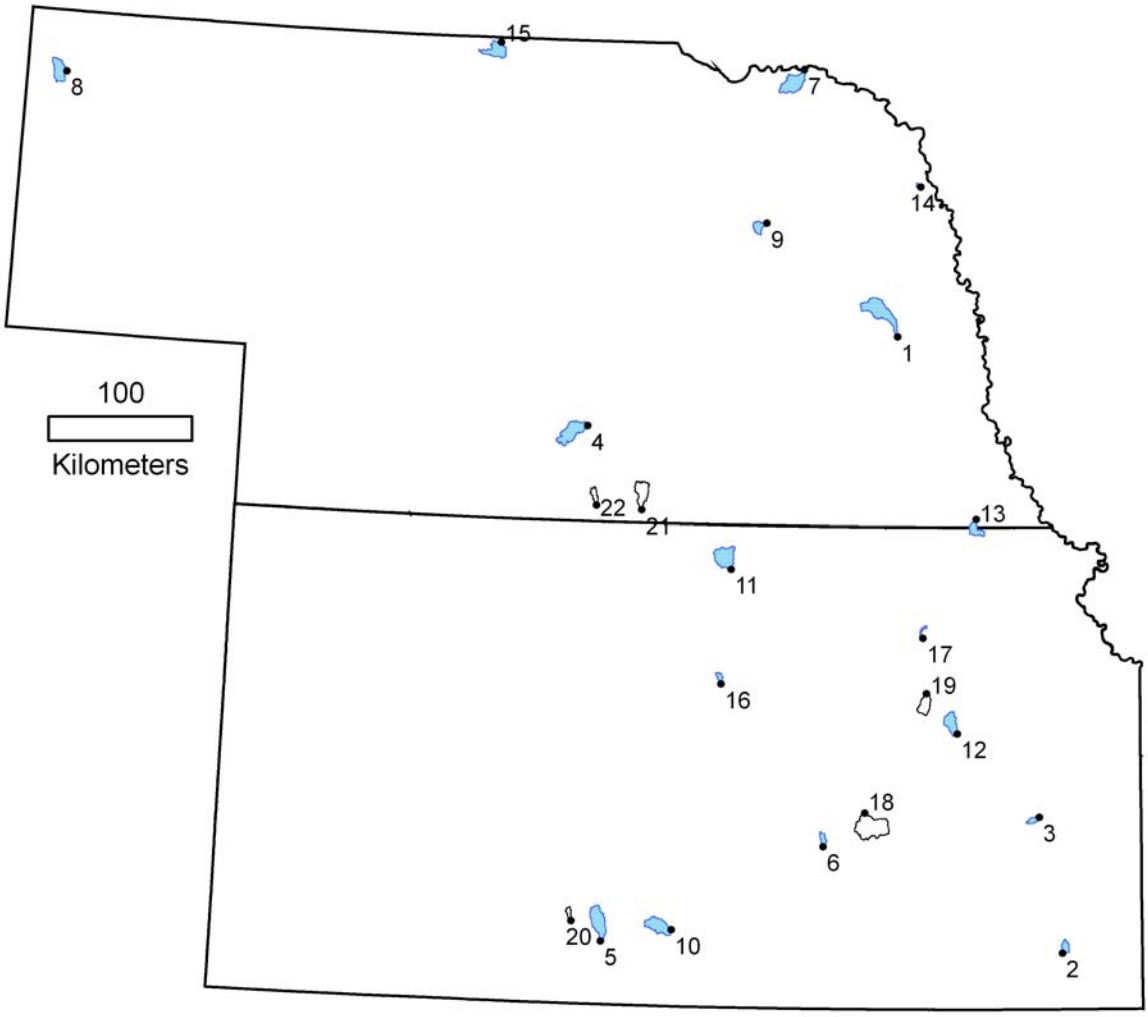

Figure 5. Location of sample watersheds.
Watersheds were delineated based on sampling location and existing synthetic stream network data generated by the Kansas Applied Remote Sensing Program of the Kansas Biological Survey. Delineated watersheds are indicated to scale by closed irregular polygons. Filled (blue) polygons indicate probability sites, while empty (white) polygons indicate best professional judgment reference sites.

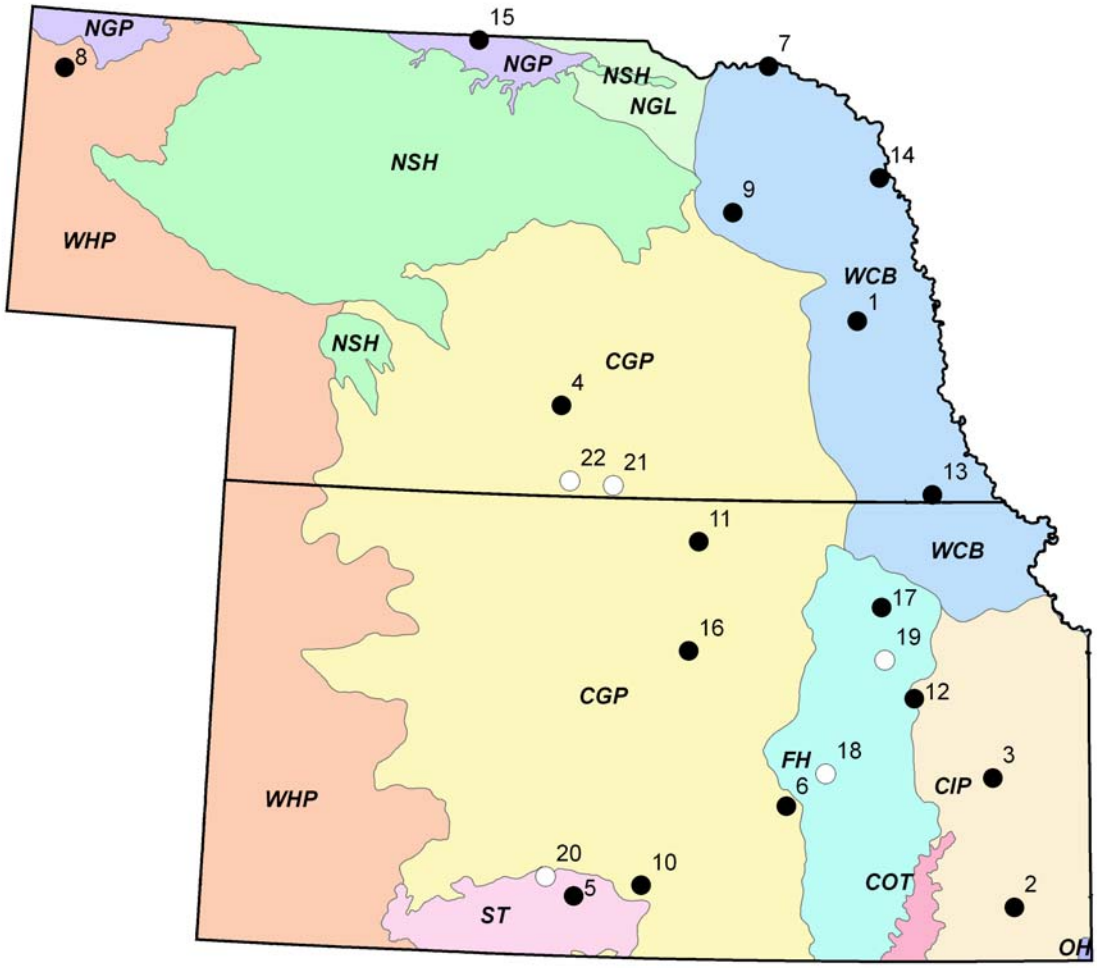

Figure 6. Sampling locations by Omernik Level III Ecoregion.
Open circles indicate reference condition sites selected by best professional judgment, while filled circles indicate probability-selected, non-reference sites. NGP: Northern Great Plains; WHP: Western High Plains; NSH: Northern Sand Hills; NGL: Northern Glaciated Plains; WCB: Western Corn Belt Plains; CGP: Central Great Plains; ST: Southwest Tablelands; COT: Central Oklahoma Tablelands; CIP: Central Irregular Plains; OH: Ozark Highlands.

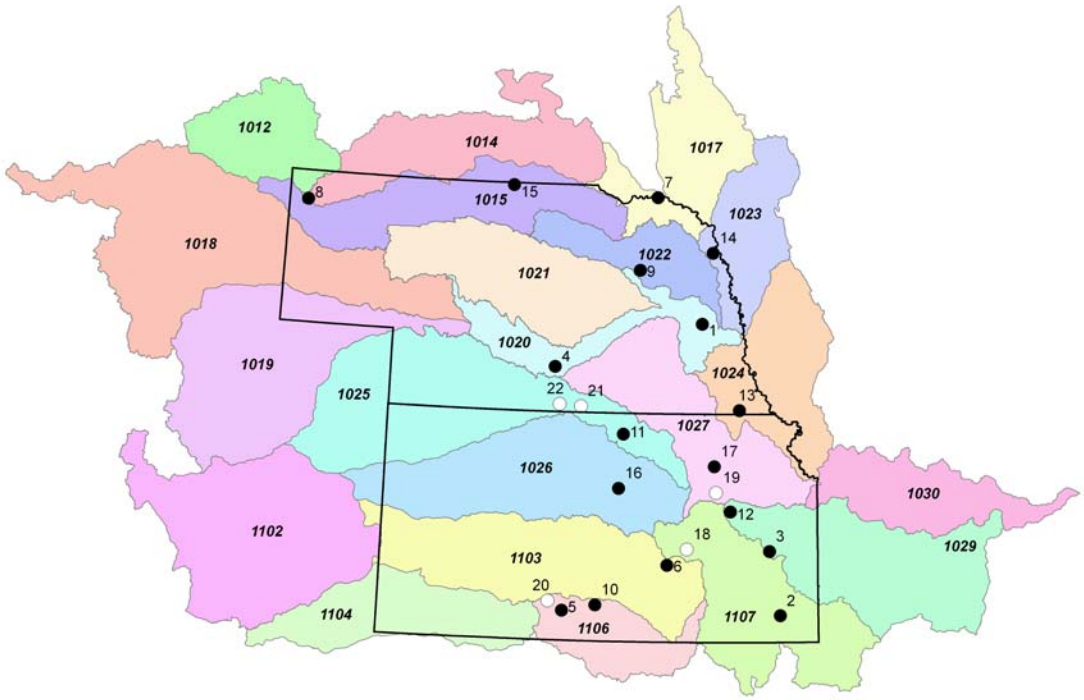

Figure 7. Sampling locations by US Geological Survey (USGS) four-digit hydrologic unit codes (HUC4) for the Missouri River basin.
Open circles indicate reference condition sites selected by best professional judgment, while filled circles indicate probability-selected, non-reference sites.

Methods

Each site was sampled once between June and August of 2004 (see Appendix A – Selected Watershed and Site Characteristics for specific sampling dates), and samples were processed within 60 hrs of collection, with the exception of total gene count data. Total gene count data were recovered later (summer 2005) from samples that had been kept frozen at -80ºC, once the need for normalization of resistance genes to 16S-rRNA counts became apparent. Tetracycline and tetracycline resistance gene samples were collected by the author (10 sites) and Tony Stahl (12 sites) of

KDHE. Additional field data were collected by CPCB (10 sites) and KDHE (12 sites) personnel. Funding for field collection was provided by USEPA as part of the WSA ("Assessment of Wadeable Streams within the South Central Semi-Arid Prairies Ecoregion using an EMAP Randomized Study Design," FED36940, X7-83177001). Laboratory processing of samples was performed both by the author (tetracyclines) and Chuck Knapp (resistance genes) of KU, with funding provided by USEPA (Grant CP-98722801-0). Additional data were collected and processed as described below.

Field Methods

At each stream site, a stream length of 40 times the average of wetted widths was delineated as the study reach. The study reach was subsequently divided into ten sections via 11 transects (A through K, downstream to upstream), according to standard WSA procedures (Figure 8) (USEPA 2004b).

One sample for tetracyclines and three samples for resistance genes were taken at each of three locations (downstream, middle, and upstream) within the stream reach (Figure 9). Samples for both tetracyclines and resistance gene analysis were taken six inches below the water surface at the center of the stream channel. Latex gloves were worn when taking and handling field samples to avoid contamination, and sample containers were rinsed three times with native water before sample collection. Samples were always taken upstream of rinsing and other concurrent sampling activities. Tetracycline samples were collected in one autoclaved 500mL amber glass jar per location. Resistance genes were collected in

three sterile 50 mL centrifuge tubes per location. Once collected, samples were placed immediately on dry ice and kept frozen until laboratory processing.

Additional data on study reaches, including physical habitat, water chemistry, and benthic macroinvertebrate samples, were collected using the methods outlined in the Wadeable Streams Assessment Field Operations Guide (USEPA 2004b).

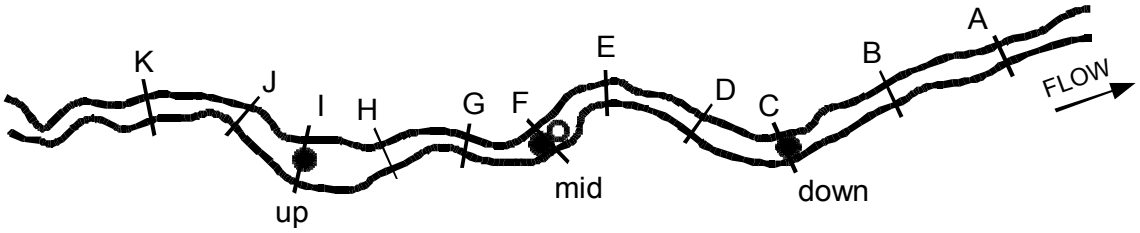

Figure 8. Schematic sampling reach with transects, typical gene sample locations (closed circles) and typical tetracycline sample location (open circle). The length of a sampling reach is 40x its width, and the eleven transects (A – K) are equally spaced with 4x the stream width between each of them. Sampling moves from downstream (A) to upstream (K) to minimize contamination by the field crew.

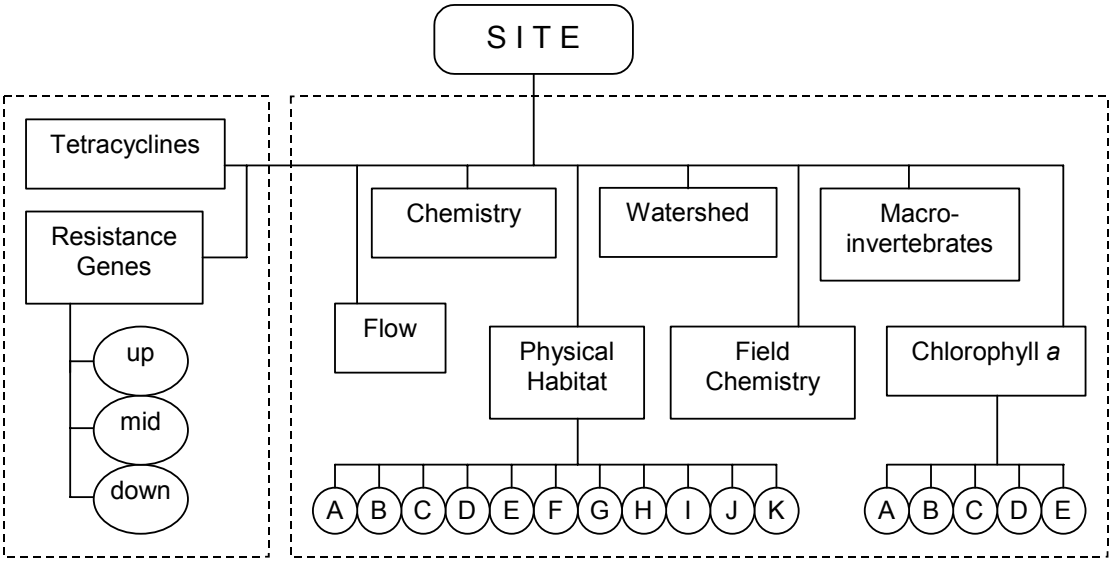

Figure 9. Schematic of data collected at each site.
The portion of the data in the dashed box to the right is standard for each site included the Wadeable Streams Assessment (USEPA 2004a; USEPA 2004b; USEPA 2004c; USEPA 2004d; USEPA 2006). Indicator groups were sampled either as composite for the whole site (e.g., Flow, Chemistry, etc.), or by transect (physical habitat and chlorophyll *a*). Tetracyclines and resistance genes data (the dashed box to the left) were added to 22 specific sites for this study. Some additional watershed data were also required for this study (e.g., CAFO locations and animal unit processing).

Laboratory Methods

Tetracyclines

Tetracycline samples were thawed and passed through a 0.7 micron Whatman GF/F glass fiber filters to remove solids. The filtrate was thoroughly mixed, and two representative, 50 µL aliqouts per sampling location were placed in reading wells of a RIDASCREEN® Enzyme-lined immunosorbent assay (ELISA) sampling kit (R-Biopharm, Darmstadt, Germany). RIDASCREEN® Tetracyclin is an enzyme immunoassay that provides quantitative analysis of tetracyclines as a class, and

thereby a general assessment of tetracycline levels in the water column (Aga et al. 2003).

Extraction of tetracyclines is known to be affected by dissolved organics and matrix effects (Lindsey et al. 2001). For example, Aga et al. (2003) observed that ELISA determination of relatively high concentrations of tetracyclines (approximately 20,000 ppb) was affected in liquid cattle manure having approximately 100 mg/L of dissolved organic matter. However, a dilution of at least 1:8 (i.e., reduction of dissolved organic matter concentration to about 100/8 or 12.5 mg/L) allowed tetracycline recovery of known samples to over 95%, thereby eliminating matrix effects. Further, dilutions of 1:100 were required to bring tetracyclines within the range of the ELISA (Aga et al. 2003). Samples in this study were not diluted, since ambient levels of both tetracyclines and of dissolved organic matter were expected to be relatively low. As expected, the dissolved organic carbon concentrations of the respective samples for this study were < 12.6 mg/L for all sites (see Appendix A – Selected Watershed and Site Characteristics), suggesting sufficient dilution to avoid matrix effects observed by Aga et al (2003).

Tetracycline concentrations were determined by spectrophotometry for each of the aliquots using RIDASCREEN® proprietary equipment and software (R-Biopharm, Darmstadt, Germany). An average absorbance value was determined for each sampling location from the resulting two measurements, and the absorbance values compared to a previously generated standard curve by RIDA® proprietary software to provide equivalent measurements of total tetracyclines in parts per trillion

(ppt). Standard solutions (50, 150, 450, 1350, 4050 ppt) were used to calibrate the standard curve.

Resistance Genes (tetW, tetQ, and tetO)

The ribosomal protection genes *tetW*, *tetQ*, and *tetO* were selected as indicators of tetracycline resistance. Although additional tetracycline resistance determinants are known, these three are among the most mobile, and the mechanisms of their horizontal transfer between and among organisms are relatively well understood. Moreover, these genes have been found in livestock waste, human waste, and environmental samples, with *tetW* being among the most commonly observed of *tet* determinants. Development of *tetW* primers in work concurrent with this study are reported in Smith et al. (2004b).

For each reach location (downstream, middle, upstream), samples were thawed and thoroughly mixed. Three 2 mL aliquots were collected from each sample (one for each of tetW, tetQ, and tetO analysis, respectively). Aliquots were centrifuged at 20,000 g for 10 minutes, then the supernatant removed, and the solids frozen at -80ºC for extraction of DNA.

DNA extraction

For DNA to be isolated and enumerated, it must first be extracted from the cells and material present in the sample. Typically, DNA extraction is achieved by a lysing process to break open cell membranes, followed by a solvent-mediated phase removal of genetic material. DNA extractions were performed as described by Smith et al. (2004b) using UltraClean DNA extraction kits supplied by Mo-Bio (Mo-Bio

Laboratories, Inc., Carlsbad, CA). Manufacturer's suggested protocols for the lysis of Gram-positive bacteria (10 minute incubation of samples at 70 ˚C) and high-yield DNA were followed. A ribolyzer (Qbiogene; 45 sec) was used to facilitate cell lysis, and DNA extracts were stored at -20 ˚C.

Real-time PCR Quantification

Once the genetic material has been extracted, it must be amplified to provide a discernible signal. Real-time polymerase chain reaction (PCR) is the most common method for this amplification and can multiply DNA molecules up to 9 orders of magnitude in a relatively short time. Known gene sequences called primers are added in very high concentrations to heat denatured DNA to mark the gene of interest. Since the primers are in such high concentrations relative to the denatured fragments of the target gene, these target fragments tend to anneal to the primers, rather than annealing back to themselves. This annealing process occurs as the solution cools. DNA polymerase is then added to extend the primers, using the target strands as the template. After a sufficient time for extension to occur, the mixture is heated to denature the newly extended strands, and the process is repeated. In this manner, small amounts of DNA from the initial sample can be replicated into much larger amounts. These larger amounts provide an amplified signal for the targeted genes.

The probe/primer sets used for tetW, tetQ, and tetO were those developed by Smith et al. (2004b), and 16S-rRNA probe/primers were adapted from Harms et al. (2003). A Bio-Rad iCycler Detection System and Taqman Universal PCR Master Mix (Smith et al. 2004b) were used for PCR amplifications. Further details on the

procedure and quality assurance can be found in Smith et al. (2004b). To conserve limited amounts of available primer material, 25 uL reactions (half that of Smith et al. 2004b) were used with primer concentrations of 900 nM for tetW and tetO and 300 nM for tetQ, respectively. Three μL of sample were added as a DNA template. Reactions were run using a Bio-Rad iCycler programmed with the following cycle conditions: 94 ºC for 10 min (DNA denaturation) with 40 cycles consisting of 60 ºC for 30 sec (primer annealing and elongation) followed by 94 ˚C for 15 sec (denaturation). Standards were prepared by serially diluting (10^8 to 10^1 copies/ μL) plasmids, each containing a *tet* resistance gene, of a known quantity. To check for interference from matrix effects, samples were spiked with known amounts of DNA template and the concentrations compared. Serial dilutions of samples and diluted plasma controls were also compared. No significant interference was observed.

Additional Analytes

Processing of additional analytes, including total suspended solids (TSS), nutrients, metals, and pH, were performed within 60 hrs of collection by the National Health and Environmental Effects Research Laboratory of the USEPA Western Ecology Division (Corvallis, OR). Sample collection, shipping, processing, laboratory analysis, and reporting were carried out as part of the WSA (USEPA 2004b; USEPA 2004c; USEPA 2004d).

Data and Data Sources

Data used for this project were obtained both from relational and geospatial databases and were collected from multiple sources (Table 1). Electronic data were collected either by standard correspondence (Nebraska CAFOs data), electronic correspondence (Kansas CAFOs data), direct download from the internet (Administrative Boundaries), or direct access to electronic files (all other data). Data processing and preparation for statistical and geospatial analysis was performed using Microsoft Access® 2000 (Microsoft Corporation).

Statistical Data Processing

The bulk of statistical processing, including data exploration, transformation of variables, analysis of variance, correlation, and graphing, was done using NCSS® 2004 (Hintze 2004). Cumulative distribution functions were produced using the spsurvey library of R statistical software, version 2.4.1 (R Development Core Team 2009) and a novel script based on previous work by Tony Olsen (personal communication). Additional graphs were produced via Sigmaplot® 8.0 (Systat Software, Inc.). Where appropriate, data were log10 transformed to satisfy distribution assumptions of parametric statistical tests (linear models, including regressions, correlations, and analysis of variance).

Geospatial Data Processing

Relational data were processed for inclusion with geospatial data using Microsoft Access® 2000 (Microsoft Corporation) and ArcGIS® 9.1 (ESRI, Inc.). Kansas animal units, a relative scale developed by KDHE for regulatory use in

livestock operations (KDHE 2009), were calculated and summed by Public Land Survey System (PLSS) Section for both Kansas and Nebraska. Unique identifiers were also developed for each PLSS Section to relate to PLSS geospatial data. Animal units were joined with PLSS layers in ArcMap® (ArcGIS® 9.1) using this unique Section identifier. Sampling sites were imported to ArcMap® and projected to match previously existing geospatial data from the Kansas Applied Remote Sensing Program (KARS) of the Kansas Biological Survey.

Geospatial data were processed using ArcView® 9.1 and ArcInfo® 9.1 (ESRI, Inc.). Since the majority of existing geospatial data available from KARS were in the Albers Equal Area (NAD 83) projection, all data were eventually projected to match these data for analysis. Raster files were developed based on 30m x 30m cells, again to match existing geospatial data for analysis. Existing files were also divided by 4-digit HUC. Therefore, sampling sites were processed by HUC as well. Synthetic stream networks, flow accumulation grids, and flow direction grids for Kansas and Nebraska had been previously developed by KARS with funding from the US Environmental Protection Agency, Region VII (KARS 2005). Using existing synthetic networks and flow accumulation grids, watersheds for each site were delineated using standard ArcInfo tools. Pour point snapping and watershed delineation were performed once manually to confirm the process, then batched using an AML script for the remaining HUC basins. Watershed areas and additional watershed characteristics were calculated using the delineations generated by this method.

Additional geospatial data were developed by USEPA using methods published for the WSA (USEPA 2004b).

Table 1. Summary of geospatial data and data sources.

	Data Set	Source Agency[1]	Data Format[2]	Citation
Relational Data[3]				
	CAFO[4] data for Kansas	KDHE	xls	(KDHE 2006)
	CAFO[4] data for Nebraska	NDEQ	xls	(NDEQ 2006)
	Sampling Site Data (latitude/longitude recorded with GPS using WGS 1984)	CPCB	mdb	collected by author
	WWTP[5] data for Kansas	KDHE	xls	(KDHE 2006)
	WWTP[5] data for Nebraska	NDEQ	xls	(NDEQ 2006)
	Additional Sample Data	USEPA	mdb	(USEPA 2006)
Geospatial Data				
	Public Land Survey System (PLSS) for Kansas (NAD 83)	DASC	geodatabase	(DASC 2000)
	PLSS for Nebraska (NAD 83)	NDEQ	coverage	(NDEQ 2000)
	Synthetic Stream Network	KARS	raster	(KARS 2005)
	Flow Accumulation Grid	KARS	raster	(KARS 2005)
	Flow Direction Grid	KARS	raster	(KARS 2005)
	Kansas Boundary 2000 (NAD 83)	Census	shp	(US Census Bureau 2001)
	Nebraska Boundary 2000 (NAD 83)	Census	shp	(US Census Bureau 2001)

[1] Data source agencies:
Kansas Department of Health and Environment, Bureau of Water, Livestock Waste Management Division (KDHE); Nebraska Department of Environmental Quality (NDEQ); Kansas Biological Survey, Central Plains Center for BioAssessment (CPCB); United States Environmental Protection Agency (USEPA), Data Access and Support Center (DASC); Kansas Biological Survey, Kansas Applied Remote Sensing (KARS); US Department of Commerce, Census Bureau, Geography Division, Cartographic Products Branch (Census)

[2] Data formats:
Microsoft Excel 2000 spreadsheet (xls); Microsoft Access 2000 database (mdb); ArcGIS 9.1 geodatabase (geodatabase); Arc/INFO coverage (coverage); ArcGIS 9.1 raster file (raster); ArcGIS 9.1 shapefile (shp)

[3] Relational data were imported from Microsoft Excel spreadsheets into a Microsoft Access 2000 database. Data manipulations, including animal unit conversions, animal units by section, and unique section identifiers were developed in this database for relation to the PLSS layers for Kansas and Nebraska.

[4] Confined Animal Feeding Operation (CAFO)

[5] Wastewater Treatment Plant (WWTP)

Q1: What are the Baselines for Tetracyclines, Total Gene Counts, and Tetracycline Resistance in the Environment?

Significant concern has arisen regarding a widespread increase in antibiotic resistance due to anthropogenic disturbance (Seveno et al. 2002; Smith et al. 2004a; Speer et al. 1992), but some antibiotic compounds (e.g., tetracyclines) and their resistance genes do occur naturally (Chopra and Roberts 2001). With this rising concern, the ability to detect changes in the incidence of resistance genes is of great importance. However, without an established baseline for comparison it is impossible either to discern between anthropogenic and natural impacts or to detect shifts in incidence. Many targeted studies have collected data in impaired sites, but relatively few have considered control or non-impacted sites, and even fewer (one for tetracyclines (Kolpin et al. 2002) and none for resistance genes to this author's knowledge) have directly examined the widespread incidence of tetracyclines or resistance genes in the environment. The first goal of this study is to identify this baseline or ambient background condition for tetracyclines, total gene counts, and tetracycline resistance genes.

Since tetracyclines are a large family of compounds, each with antibiotic properties, any member of the family could provide pressure for selection of resistance. Therefore, to be conservative, the whole family of tetracyclines has been combined into one analyte for this study (referred to as "tetracyclines" for the remainder of this study, unless otherwise noted), regardless of the particular compound, degradation product, or epimer. In this way, the total amount of any

potentially active tetracyclines can be identified simultaneously. Ambient tetracycline levels were expected to be low to nonexistent, for reasons both physiochemical (i.e., their photolytic nature and the ambient pH and temperature conditions of natural stream waters, which promote tetracycline degradation) and biological (i.e., the vast majority of naturally occurring microbes identified to date do not produce tetracyclines). Evidence for such rapid degradation of tetracyclines at nominal surface water conditions has been shown (Chopra and Roberts 2001; Engemann et al. 2006; Qiang and Adams 2004).

Peak et al. (2007) observed that tetracyclines and tetracycline resistance genes were significantly positively correlated in some feedlot lagoons, but Engemann et al. (2006) found that resistance gene levels were not related to tetracycline levels in mesocosm studies using cattle feedlot effluents. Whether tetracyclines were found to be correlated with resistance genes or not, the ambient levels of tetracycline resistance genes were expected to be low. This was expected for both the total abundance of resistance genes and for the abundance relative to the total amount of genes in the system. However, since many resistance genes are contained in mobile genetic elements that could be introduced from a widespread array of sources, the expectation of low environmental resistance levels was taken as an arbitrarily conservative starting point, rather than a base of previous knowledge. As a corollary, the range of resistance genes at any given site was also expected to be relatively small (since they are expected to be low), and therefore comparison of sites or groups by

the minimum, average, or maximum of observed values should yield statistically similar results.

In order to examine large-scale patterns in the distribution of both tetracyclines and tet resistance genes, the 22 sites included in this study were grouped according to four factors: state; reference condition (best professional judgment "reference" sites versus probability "non-reference" sites); Omernik Level III ecoregion (Omernik 1987); and USGS hydrologic unit (Seaber et al. 1987). State was chosen as a factor both for its interest to personnel in regulatory agencies (NDEQ and KDHE) who were to be associated with data collection, and for potential differences in antibiotic application laws or practice between Kansas and Nebraska. Reference condition was selected as a factor on the theory that less anthropogenically impacted "reference" sites might exhibit some higher level of environmental quality than more impacted "non-reference" sites (i.e., if there are differences in tetracycline or tetracycline resistance gene levels in the environment, then impacted sights might show a larger signal than non-impacted sites). Ecoregion and hydrologic unit were both selected on the theory that different ecological and hydrologic conditions might exhibit different tetracycline and tetracycline resistance profiles. For completeness, as a conservative starting point, the null hypothesis was again adopted for all four factors, and no statistical differences were expected within or among any of these groups, since the ubiquitous ambient levels of both tetracyclines and tetracycline resistance genes were expected to be low to non-existent. Similarly, no differences were expected between sites.

Observed Values and Patterns

In order to rule out potential seasonal differences in observed values, sampling date was included in analysis of variance. For all factors (i.e., state, reference condition, ecoregion, and hydrologic region) and all observed parameters (i.e., tetracyclines, total gene counts, and resistance counts), sampling date did not significantly affect the relationships described in the following sections ($p > 0.2$).

Tetracyclines

Water column total tetracyclines ranged from 78 to 548 parts per trillion (ppt), with a median value of 242 and a mean value of 238 ± 119 ppt for 22 samples (Table 2, Figure 10,). Site 15 was a high outlier (548 ppt) compared to the remaining sites (Figure 11). Since tetracyclines levels were found using ELISA, the observed values represent a conservative measure of all tetracyclines and their byproducts, including chlortetracycline, tetracycline, oxytetracycline, doxycycline, demeclocycline, minocycline, and meclocycline, as well as degradation compounds such as the anhydrotetracyclines and their epimers (Aga et al. 2003). RIDASCREEN® ELISA kits claim a mean lower detection limit of 0.05 parts per billion (ppb), or 50 ppt (R-Biopharm 2003). Using liquid chromatography with mass spectrometry, Aga et al.(2003) have confirmed detection limits (80% inhibition levels) of 0.01 ppb (10 ppt) for chlortetracycline, 0.05 ppb (50 ppt) for oxytetracycline, and 0.38 ppb (380 ppt) for tetracycline for these ELISA kits. Based on these detection limits for individual tetracycline compounds, the collective measurements of total tetracyclines for all sites were considered as quantifiable, real values (rather than non-detects).

Mean total tetracyclines were not significantly different between Kansas and Nebraska (Table 3, Figure 11), between reference and non-reference sites (Table 3, Figure 11), or between Omernik Level III Ecoregions (Table 3, Figure 12). Though no significant differences were observed between USGS hydrologic subbasins (Table 3, Figure 13), too few data points were available for any meaningful comparisons, as most (8 of 14) had only one observation, several (4 of 14) had two observations, and only a pair (2 of 14) had three observations. Additionally, no apparent large-scale spatial patterns were initially observed in the distribution of tetracyclines among sites (Figure 14a), despite the potential sources of tetracyclines and resistance genes represented by CAFOs (Figure 2) and WWTPs (Figure 3).

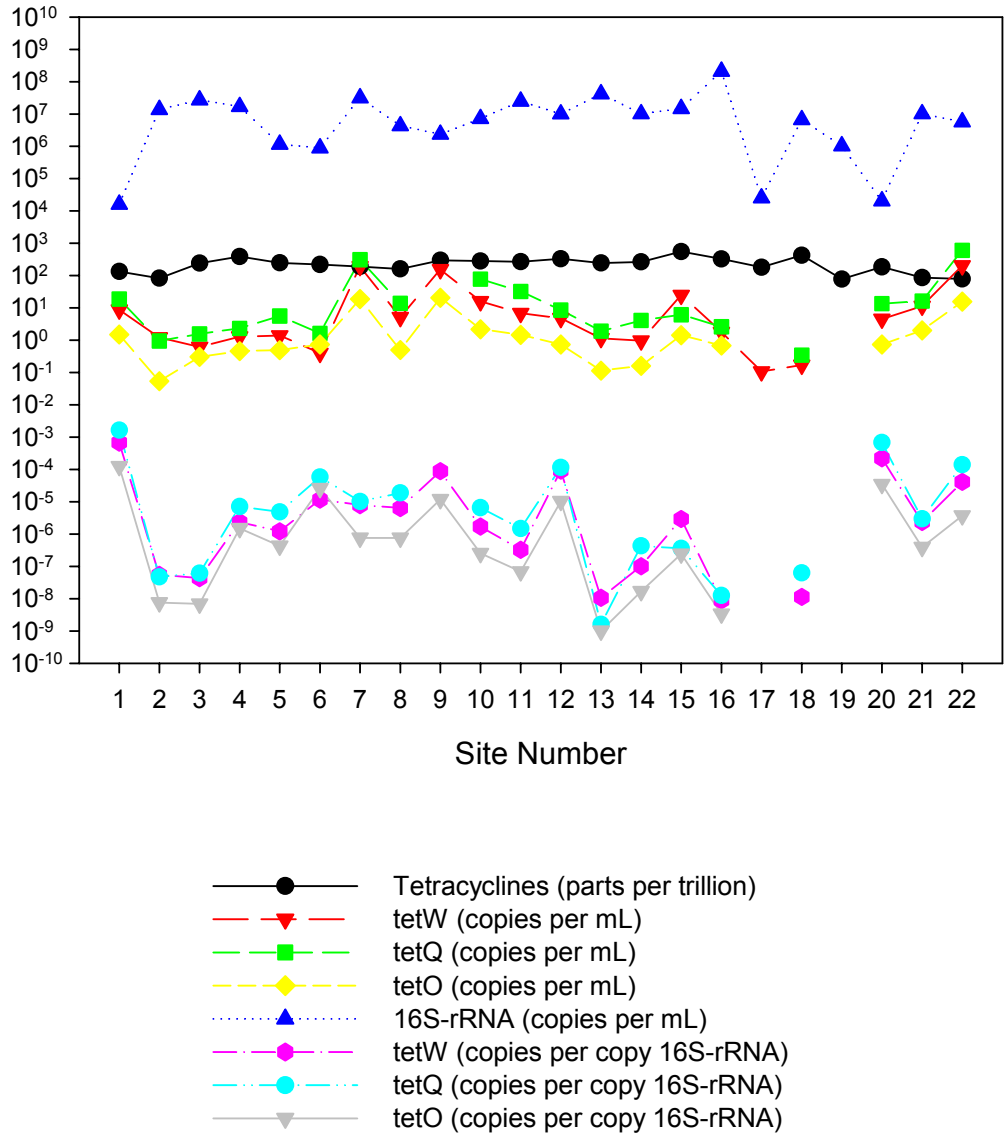

Figure 10. Observed values of tetracyclines, total gene (16SrRNA) counts, and both total gene counts and gene counts relative to 16SrRNA for tetW, tetQ, and tetO by site.

Table 2. Overall summary of observed tetracyclines, genes, and resistance genes.

Observed values are arranged as groups used for analysis: all sites combined (All); by state (KS, NE); by reference condition (Non – Nonreference, Ref – Reference); and by Omernik Level III Ecoregion (SWT – Southwest Tablelands, CIP – Central Irregular plains, WHP – Western High Plains, WCB – Western Cornbelt Plains, CGP – Central Great Plains, NGP – Northern Glaciated Plains, FH – Flint Hills). Sum of tetR indicates the sum of the observed counts of *tetW*, *tetQ*, and *tetO* combined.

		Count	*Mean*	*Standard Deviation*	*Standard Error*	*Minimum*	*Maximum*	*Median*
Tetracyclines (parts per Trillion)	*All*	22	238	119	25.4	77.8	548	242
	KS	12	237	99.1	28.6	77.8	421	243
	NE	10	239	146	46.1	78.1	548	217
	Non	17	258	106	25.8	82.9	548	246
	Ref	5	169	148	66.0	77.8	421	85.4
	SWT	1	160	0	0	160	160	-
	CIP	2	215	43.9	31.1	184	246	-
	WHP	7	234	117	44.1	78.1	387	268
	WCB	3	226	176	102	77.8	421	179
	CGP	3	217	124	71.4	82.9	327	240
	NGP	1	548	0	0	548	548	-
	FH	5	226	64.7	28.9	133	298	244
Genes (copies per mL)								
16s-rRNA	*All*	52	2.16E+07	4.82E+07	6.69E+06	8.20E+03	2.30E+08	7.16E+06
	KS	27	3.04E+07	6.47E+07	1.25E+07	1.56E+04	2.30E+08	7.14E+06
	NE	25	1.20E+07	1.50E+07	3.00E+06	8.20E+03	6.18E+07	7.28E+06
	Non	41	2.63E+07	5.34E+07	8.34E+06	8.20E+03	2.30E+08	9.35E+06
	Ref	11	3.94E+06	5.03E+06	1.52E+06	1.56E+04	1.51E+07	1.06E+06
	SWT	3	4.27E+06	4.54E+06	2.62E+06	6.07E+05	9.35E+06	2.86E+06
	CIP	4	3.03E+05	5.65E+05	2.83E+05	1.56E+04	1.15E+06	2.24E+04
	WHP	17	4.62E+07	7.79E+07	1.89E+07	2.81E+04	2.30E+08	1.01E+07
	WCB	6	3.65E+06	5.88E+06	2.40E+06	2.40E+04	1.51E+07	1.01E+06
	CGP	6	1.40E+07	9.39E+06	3.83E+06	2.07E+04	2.71E+07	1.49E+07
	NGP	3	1.46E+07	1.38E+07	7.99E+06	3.05E+06	2.99E+07	1.08E+07
	FH	13	1.33E+07	1.85E+07	5.14E+06	8.20E+03	6.18E+07	7.28E+06

Table 2 (continued). Overall summary of observed tetracyclines and genes.

		Count	*Mean*	*Standard Deviation*	*Standard Error*	*Minimum*	*Maximum*	*Median*
tetW	*All*	51	31.3	69.0	9.67	0.05	285	3.32
	KS	25	4.32	5.80	1.16	0.0458	23.1	1.86
	NE	26	57.3	89.8	17.6	0.444	285	8.51
	Non	41	23.0	57.1	8.92	0.0458	285	3.11
	Ref	10	65.3	102	32.2	0.171	278	7.11
	SWT	2	5.08	2.71	1.91	3.16	6.99	-
	CIP	4	3.71	3.78	1.89	0.966	9.16	2.35
	WHP	20	35.9	76.5	17.1	0.134	278	4.25
	WCB	2	0.138	0.0461	0.0326	0.106	0.171	-
	CGP	8	2.38	2.20	0.779	0.0458	5.68	1.78
	NGP	3	24.6	17.4	10.1	10.4	44.0	19.4
	FH	12	63.6	95.4	27.6	0.444	285	8.51
tetQ	*All*	48	69.2	173	24.9	0.07	839	6.62
	KS	24	17.4	32.4	6.61	0.138	140	5.73
	NE	24	121	233	47.6	0.0668	839	7.98
	Non	37	38.3	103	16.9	0.0668	559	5.55
	Ref	11	173	294	88.8	0.138	839	14.4
	SWT	2	13.8	10.7	7.59	6.18	21.4	13.8
	CIP	4	11.5	8.58	4.29	5.55	23.9	8.24
	WHP	19	116	232	53.3	0.155	839	14.4
	WCB	2	0.342	0.289	0.204	0.138	0.546	0.342
	CGP	8	3.94	4.04	1.43	0.424	11.4	1.72
	NGP	2	6.12	1.50	1.06	5.06	7.18	6.12
	FH	11	90.4	177	53.3	0.0668	559	8.78
tetO	*All*	46	3.45	6.57	0.969	0.0411	26.5	0.809
	KS	21	0.913	0.896	0.196	0.0411	3.43	0.743
	NE	25	5.58	8.36	1.67	0.0420	26.5	1.28
	Non	37	2.80	6.10	1.00	0.0411	26.5	0.731
	Ref	9	6.10	8.10	2.70	0.175	24.3	1.28
	SWT	2	0.496	0.558	0.394	0.101	0.890	-
	CIP	4	0.674	0.415	0.208	0.175	1.02	0.752
	WHP	19	3.56	5.99	1.38	0.126	24.3	1.11
	WCB	-	-	-	-	-	-	-
	CGP	7	0.416	0.324	0.122	0.0411	0.809	0.423
	NGP	3	1.42	0.750	0.433	0.556	1.88	1.82
	FH	11	7.29	10.0	3.02	0.0420	26.5	1.37

Table 2 (continued). Overall summary of observed tetracyclines and genes.

		Count	Mean	Standard Deviation	Standard Error	Minimum	Maximum	Median
Sum of tet^R	*All*	52	97.7	234	32.5	0.106	1141	12.4
	KS	26	20.9	37.7	7.39	0.106	165	5.63
	NE	26	174	314	61.5	0.552	1140	21.0
	Non	41	60.1	155	24.2	0.106	871	9.44
	Ref	11	238	396	119	0.309	1140	20.8
	SWT	2	19.3	14.0	9.90	9.44	29.2	19.3
	CIP	4	15.9	12.7	6.33	7.06	34.0	11.2
	WHP	20	150	307	68.5	0.1341994	1140	16.2
	WCB	3	0.320	0.221	0.127	0.106	0.546	0.309
	CGP	8	6.68	6.16	2.18	0.891	15.3	3.62
	NGP	3	30.1	20.0	11.6	16.0	53.0	21.3
	FH	12	153	266	76.7	0.552	871	24.9
Genes (copies per copy 16S-rRNA)								
tetW	*All*	43	7.50E-05	2.02E-04	3.07E-05	8.59E-10	1.03E-03	1.20E-06
	KS	21	4.62E-05	1.18E-04	2.56E-05	8.59E-10	4.73E-04	1.70E-07
	NE	22	1.02E-04	2.58E-04	5.50E-05	1.06E-08	1.03E-03	5.49E-06
	Non	36	6.87E-05	2.09E-04	1.39E-04	8.59E-10	1.03E-03	8.76E-07
	Ref	7	1.07E-04	1.68E-04	2.62E-04	1.13E-08	4.73E-04	6.19E-05
	SWT	2	6.31E-06	7.37E-06	5.21E-06	1.10E-06	1.15E-05	-
	CIP	4	1.67E-04	2.11E-04	1.05E-04	1.20E-06	4.73E-04	9.64E-05
	WHP	16	7.49E-06	1.71E-05	4.27E-06	8.59E-10	6.63E-05	4.43E-07
	WCB	1	1.13E-08	0	0	1.13E-08	1.13E-08	-
	CGP	6	4.60E-05	1.12E-04	4.58E-05	2.51E-08	2.75E-04	1.27E-07
	NGP	3	2.93E-06	2.98E-06	1.72E-06	9.62E-07	6.37E-06	1.47E-06
	FH	11	1.95E-04	3.47E-04	1.05E-04	1.06E-08	1.03E-03	8.41E-06
tetQ	*All*	41	1.88E-04	5.29E-04	8.26E-05	6.75E-10	2.52E-03	2.16E-06
	KS	21	1.17E-04	2.89E-04	6.31E-05	6.75E-10	1.23E-03	6.18E-07
	NE	20	2.63E-04	7.00E-04	1.56E-04	1.60E-09	2.52E-03	4.50E-06
	Non	33	1.64E-04	5.55E-04	3.61E-04	6.75E-10	2.52E-03	6.18E-07
	Ref	8	2.89E-04	4.17E-04	6.37E-04	9.11E-09	1.23E-03	1.40E-04
	SWT	2	1.87E-05	2.34E-05	1.65E-05	2.16E-06	3.52E-05	-
	CIP	4	5.08E-04	5.18E-04	2.59E-04	4.82E-06	1.23E-03	3.97E-04
	WHP	15	2.53E-05	5.40E-05	1.39E-05	6.75E-10	2.00E-04	2.96E-06
	WCB	2	6.27E-08	7.58E-08	5.36E-08	9.11E-09	1.16E-07	-
	CGP	6	5.74E-05	1.40E-04	5.71E-05	2.28E-08	3.43E-04	3.45E-07
	NGP	2	3.55E-07	1.63E-07	1.15E-07	2.40E-07	4.70E-07	-
	FH	10	4.92E-04	9.55E-04	3.02E-04	1.60E-09	2.52E-03	7.54E-06

Table 2 (continued). Overall summary of observed tetracyclines and genes.

		Count	*Mean*	*Standard Deviation*	*Standard Error*	*Minimum*	*Maximum*	*Median*
tetO	*All*	39	1.13E-05	3.36E-05	5.39E-06	6.07E-10	1.98E-04	2.25E-07
	KS	18	9.00E-06	1.65E-05	3.90E-06	6.07E-10	5.24E-05	1.21E-07
	NE	21	1.32E-05	4.37E-05	9.53E-06	1.01E-09	1.98E-04	4.28E-07
	Non	33	9.93E-06	3.54E-05	2.25E-05	6.07E-10	1.98E-04	6.09E-08
	Ref	6	1.86E-05	2.21E-05	4.17E-05	3.88E-07	5.24E-05	8.49E-06
	SWT	2	7.51E-07	1.01E-06	7.16E-07	3.54E-08	1.47E-06	-
	CIP	4	2.60E-05	2.43E-05	1.21E-05	4.26E-07	5.24E-05	2.56E-05
	WHP	15	2.60E-06	6.71E-06	1.73E-06	6.07E-10	2.60E-05	1.76E-07
	WCB	-	-	-	-	-	-	-
	CGP	5	6.24E-06	1.39E-05	6.21E-06	6.84E-09	3.11E-05	4.43E-08
	NGP	3	2.44E-07	3.24E-07	1.87E-07	5.16E-08	6.18E-07	6.09E-08
	FH	10	2.63E-05	6.22E-05	1.97E-05	1.01E-09	1.98E-04	6.56E-07
Sum of tet^R	*All*	44	2.59E-04	7.33E-04	1.11E-04	2.14E-09	3.74E-03	4.00E-06
	KS	22	1.63E-04	4.08E-04	8.69E-05	2.14E-09	1.76E-03	7.80E-07
	NE	22	3.54E-04	9.57E-04	2.04E-04	1.33E-08	3.74E-03	1.05E-05
	Non	36	2.28E-04	7.65E-04	1.27E-04	2.14E-09	3.74E-03	1.63E-06
	Ref	8	3.97E-04	5.94E-04	2.10E-04	2.04E-08	1.76E-03	1.85E-04
	SWT	2	2.57E-05	3.17E-05	2.24E-05	3.30E-06	4.82E-05	-
	CIP	4	7.01E-04	7.47E-04	3.74E-04	6.45E-06	1.76E-03	5.19E-04
	WHP	16	3.37E-05	7.23E-05	1.81E-05	2.14E-09	2.73E-04	2.71E-06
	WCB	2	6.84E-08	6.78E-08	4.80E-08	2.04E-08	1.16E-07	-
	CGP	6	1.09E-04	2.65E-04	1.08E-04	4.79E-08	6.49E-04	5.00E-07
	NGP	3	3.41E-06	3.10E-06	1.79E-06	1.48E-06	6.98E-06	1.77E-06
	FH	11	6.66E-04	1.30E-03	3.93E-04	1.33E-08	3.74E-03	1.74E-05

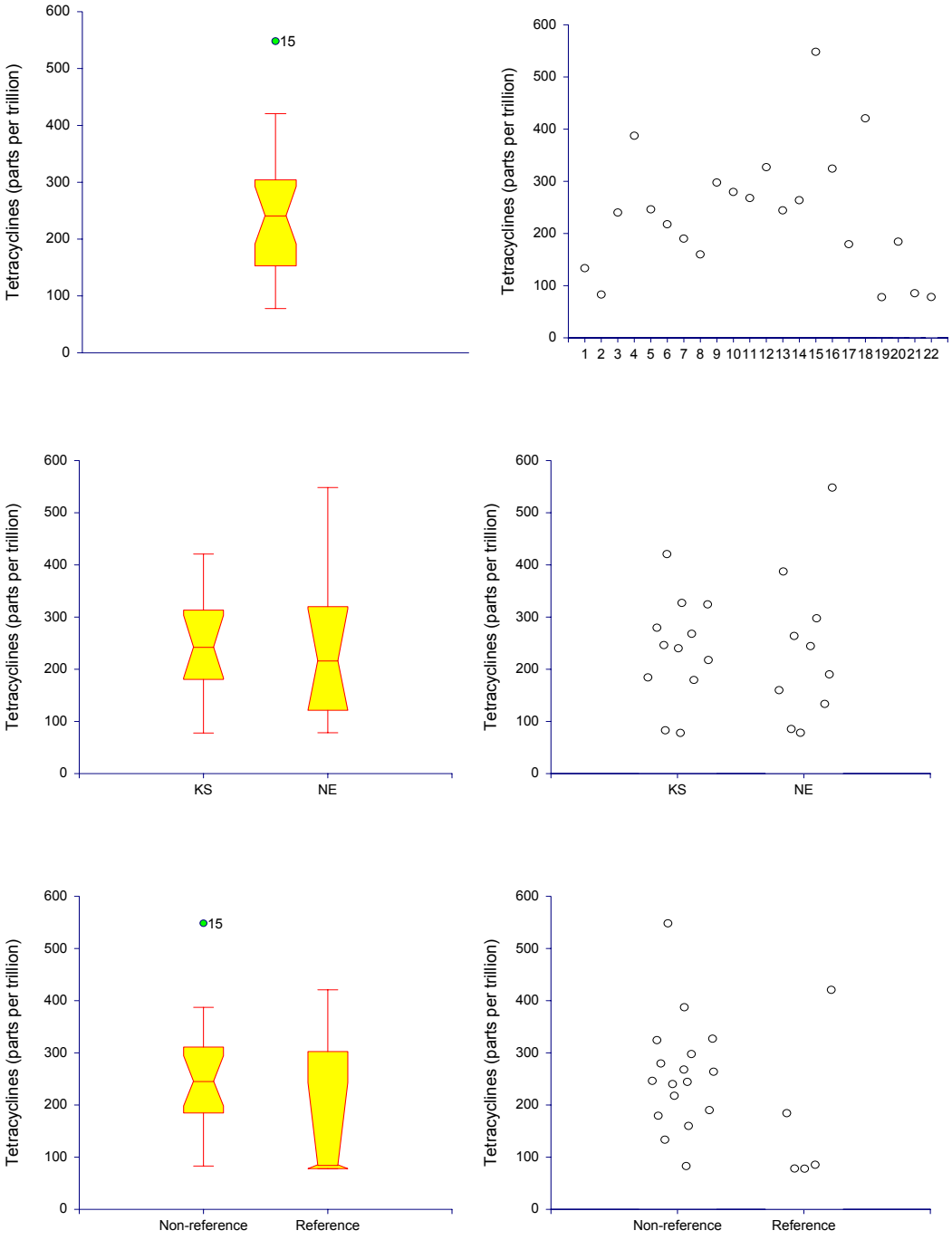

Figure 11. Summary plots for total tetracyclines by state and reference category.
Box plots (a) for all measurements, (b) by state, and (c) by reference category; and dot plots (d) for all measurements, (e) by state, and (f) by reference category.

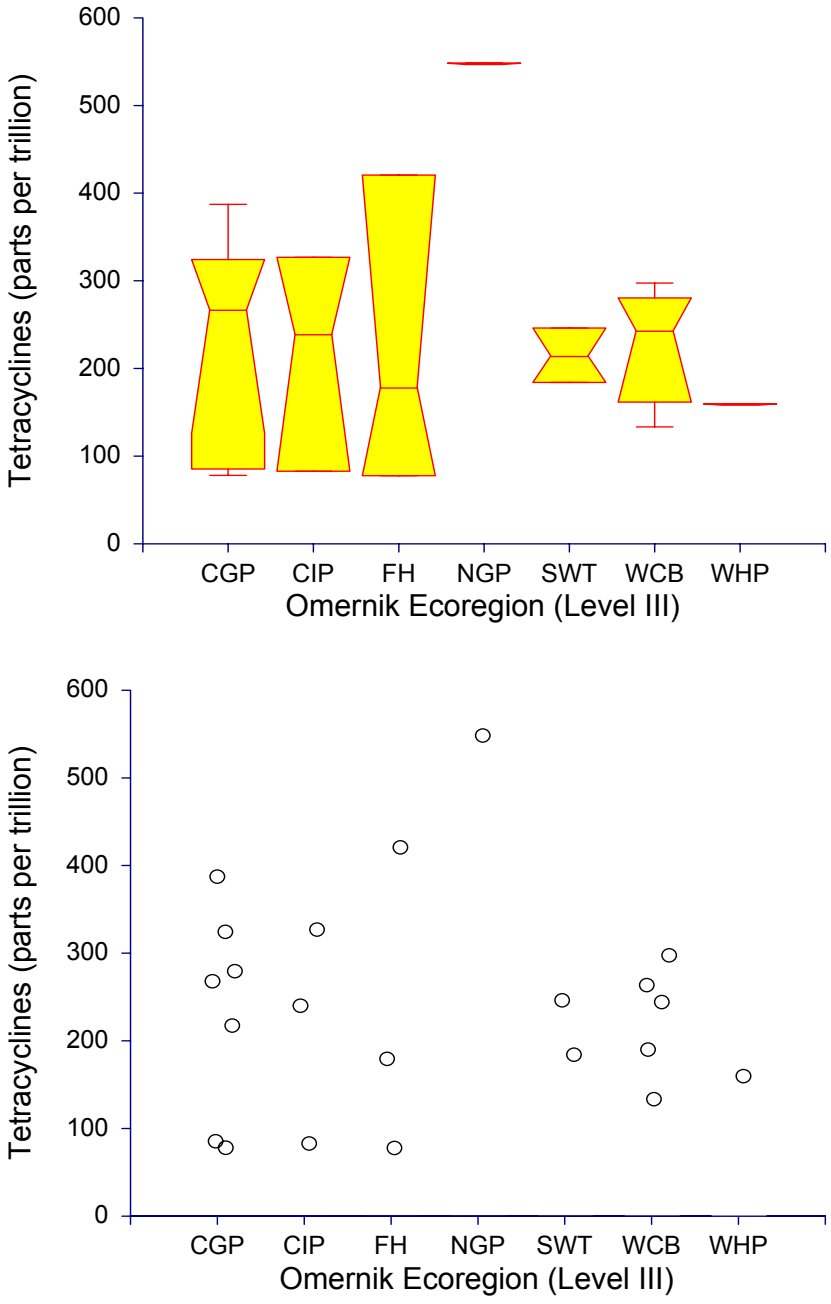

Figure 12. Box plot and dot plot of total tetracyclines by Omernik Level III Ecoregion.
NGP: Northern Great Plains; WHP: Western High Plains; NSH: Northern Sand Hills; NGL: Northern Glaciated Plains; WCB: Western Corn Belt Plains; CGP: Central Great Plains; ST: Southwest Tablelands; COT: Central Oklahoma Tablelands; CIP: Central Irregular Plains; OH: Ozark Highlands.

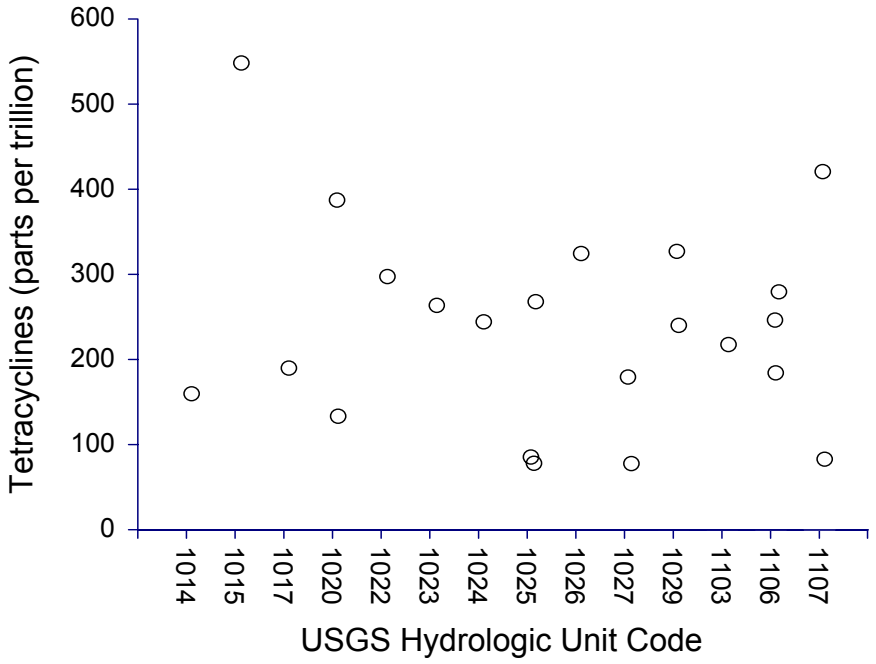

Figure 13. Dot plot of total tetracyclines by 4 digit USGS Hydrologic Unit Code.

Table 3. P-values from analysis of variance for site averages of tetracyclines, total genes (16s-rRNA), and resistance genes.
Total abundance is indicated by *tetX*, and proportion relative to 16s-rRNA count is indicated by *tetXnorm*, where X is the *tet* determinant (i.e., W, Q, or O). P-values less than 0.05 are indicated by an asterisk.

	State	Reference	Ecoregion	Hydrologic Unit
Tetracyclines	0.978	0.147	0.287	0.623
log(16s-rRNA)	0.654	0.184	0.416	0.468
log(tetW)	0.026*	0.305	0.549	0.252
log(tetQ)	0.165	0.365	0.743	0.183
log(tetO)	0.222	0.187	0.750	0.184
log(tetWnorm)	0.198	0.163	0.516	0.544
log(tetQnorm)	0.392	0.224	0.574	0.339
log(tetOnorm)	0.682	0.044*	0.626	0.606
log(SumTetR)	0.046*	0.404	0.446	0.164
log(SumTetRnorm)	0.224	0.241	0.430	0.466

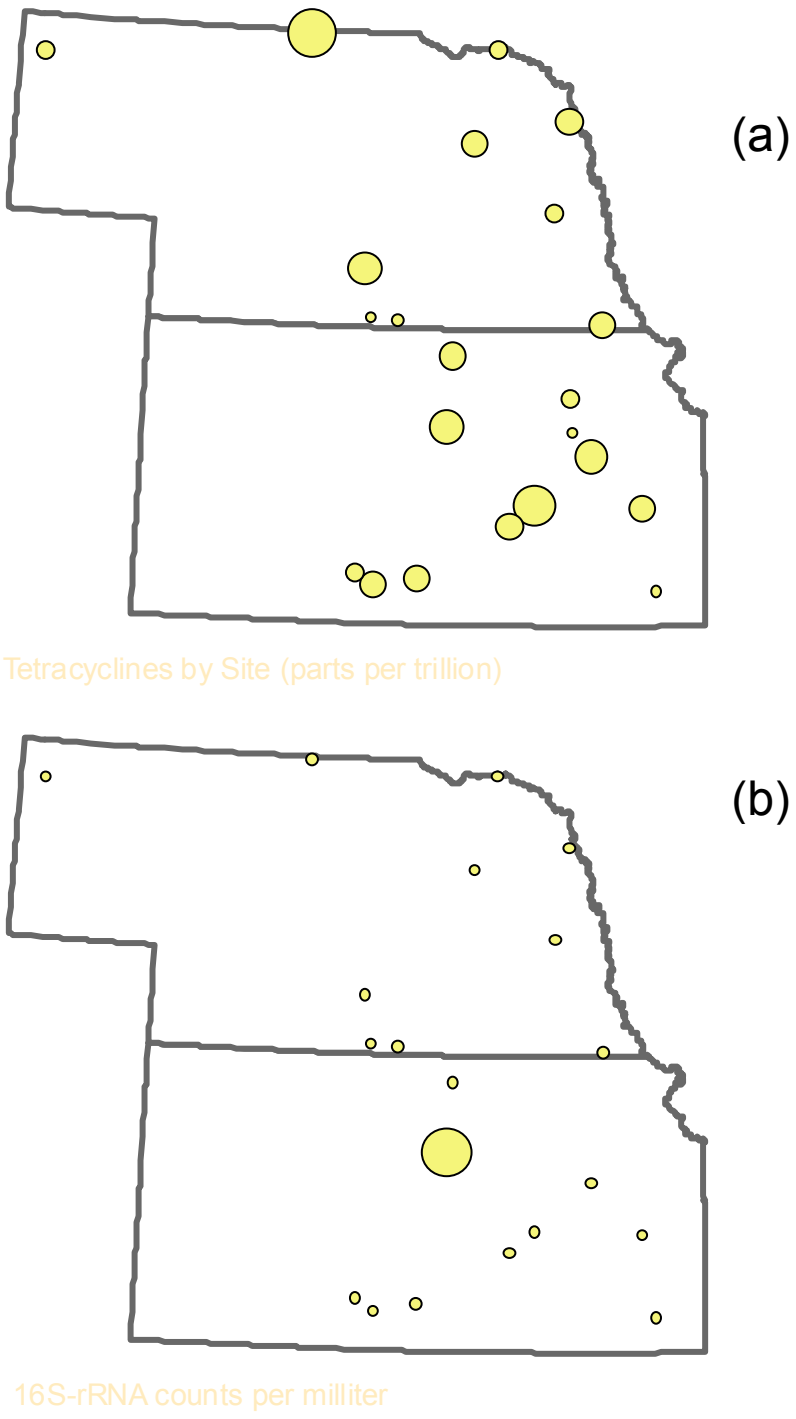

Figure 14. Spatial representation of observed average (a) total tetracyclines (parts per trillion) and (b) total gene counts (copies of 16S-rRNA per mL). Relative sizes of symbols indicate quartile distribution of values.

Total Numbers of Genes

Previous studies have shown 16S-rRNA can be used as a measure of the total number of genes present in each water column sample (Harms et al. 2003; Lee et al. 2002). Extracted, enumerated total gene counts include both free RNA strands and fragments from lysed cells. Though all of these genes may not be viable nor activated *in vivo*, counts obtained by this method are considered a conservative estimate of the total number of genes in a given sample (Fitch and Margoliash 1967). Where possible, total gene counts were measured for each of the three samples (down, middle, up) taken at each site, and then used to calculate the average total gene counts, minimum total gene counts, and maximum total gene counts for each site.

Observed average total gene counts in the water column ranged from 8.2×10^3 to 2.3×10^8 copies per milliliter, with a median value of 7.16×10^6 copies per mL and a mean of $2.16 \times 10^7 \pm 4.8 \times 10^7$ copies per mL in 52 samples (Table 2, Figure 10),. Average total gene counts were highly variable with significant differences ($p<0.001$) between sites. Site 1 (8.2×10^3 copies per mL) was a low outlier for all sites combined, and Site 17 was a low outlier for non-reference sites (see Appendix C – Total Gene Counts Observed Values Figures for additional figures). No significant differences between states or reference condition were observed (Table 3). Similarly, though the Central Great Plains and Central Irregular Plains sites appeared to have higher average total gene counts than the Southwestern Tablelands, analysis of variance revealed no statistical differences between ecoregions (Table 3). No significant differences were observed in average, minimum, or maximum total gene

counts between states, between reference conditions, between ecoregions, or between hydrologic units. Similarly, no large-scale spatial patterns in observed total gene counts were apparent (Figure 14b).

Resistance Genes

Water column counts of resistance genes (tetW, tetQ, and tetO) were obtained in three samples (down, middle, up) for each site. As with total gene counts, average, minimum, and maximum values were calculated for each site. In addition, resistance gene counts were reported both directly as total abundance (i.e., as copies per mL) and as relative proportions of total gene counts (i.e., as copies per copy 16S-rRNA). Significant differences between sites were observed for each of the three *tet* genes, both in terms of total abundance and in terms of relative proportion. However, no large-scale spatial pattern was discernible in the relative proportion of *tet* genes (Figure 15, Figure 16), other than the tendency for the sites to have similarly high or low values for all three resistance genes (Figure 10).

Smith et al. (2004b) found that the $\log_{10}$ of volatile suspended solids (VSS) was proportional to the $\log_{10}$ of the sum of *tetW*, *tetQ*, and *tetO* copies in their samples (r^2 =0.43, p=0.003). The original intent of normalization of *tet* genes by VSS was to account for presumably increasing numbers of total genes with increasing organic substrate concentrations (i.e., increasing VSS). For example, consider a constant number of resistance genes. If the number of total genes is low, then the proportion of resistance genes to total genes will be high. However, if the number of total genes is high, then the proportion of that same constant number of resistance

genes will be low. Smith et al. (2004b) used VSS as a surrogate indicator for total genes in order to describe their observed tet levels as a proportion of the total gene count present. Unfortunately, VSS was not measured in this study. However, the levels of total suspended solids (TSS) observed in this study, of which VSS is a portion, were both significantly lower than the mean TSS value (41.3 mg/L) of observations from a recent study by the state of Kansas ($p < 0.01$) (Huggins et al. In Press), and observably lower than the VSS values (approximately 300 to 10,000 mg/L) reported by Smith et al. (2004b). Given the low TSS values observed in this study, inhibition of total gene counts by VSS was deemed unlikely, and since total genes (as copies of 16S-rRNA) were measured directly in this study, relative proportions of resistance genes were calculated, rather than using VSS as a surrogate as Smith et al. (2004b) did.

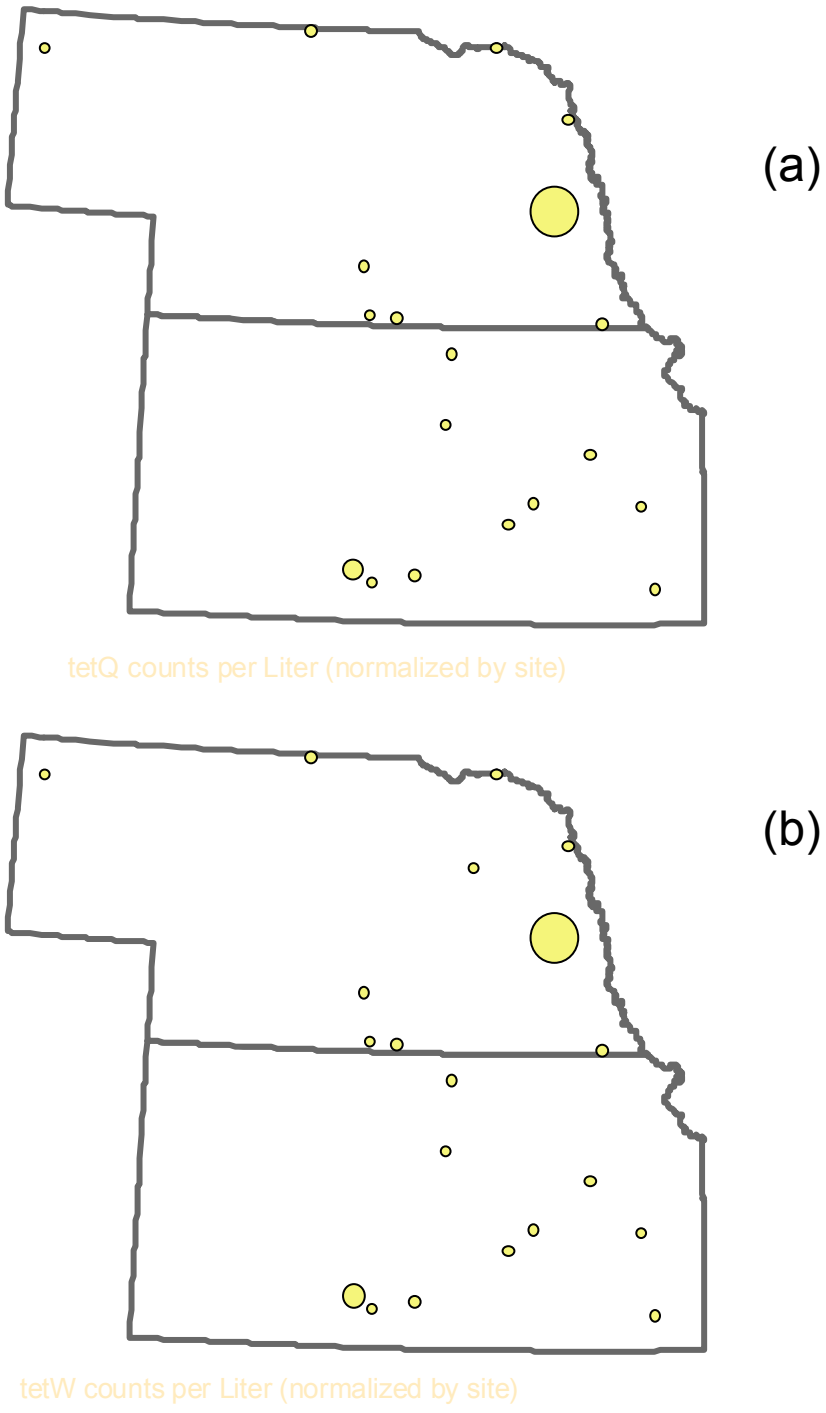

Figure 15. Spatial representation of observed average (a) tetW (copies per copy 16S-rRNA) and (b) tetQ (copies per copy 16S-rRNA).
Relative sizes of symbols indicate quartile distribution of values.

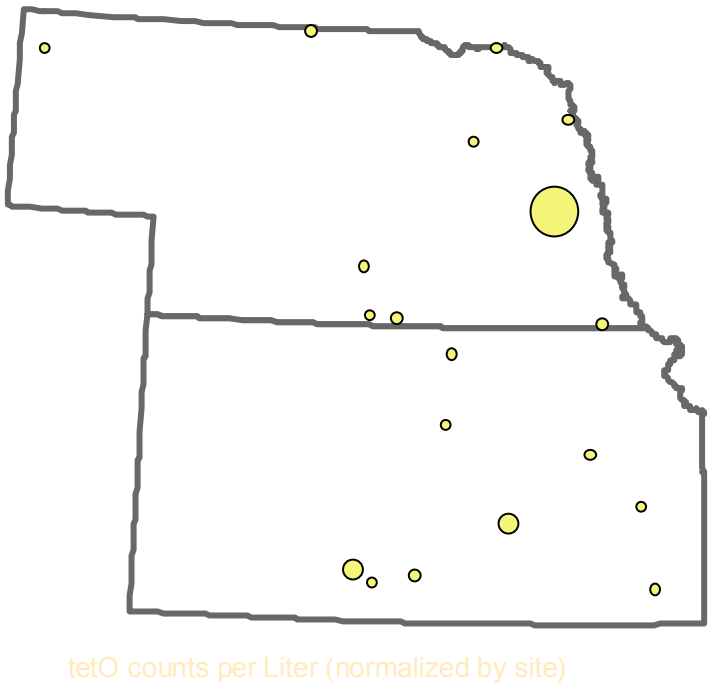

Figure 16. Spatial representation of observed average tetO (copies per copy 16S-rRNA).
Relative sizes of symbols indicate quartile distribution of values.

tetW

Total Abundance

Observations of average tetW counts ranged from 0.05 to 285 copies per mL, with a median value of 3.32 and a mean value of 31.3 ± 69 copies per mL in 51 samples (Table 2, Figure 10, Figure 17). Observed total abundances of tetW were significantly higher in Nebraska for average ($p=0.013$), minimum ($p=0.004$), and maximum ($p=0.022$) calculated values.

Relative Proportion

Observed proportional values of average *tetW* ranged from 8.6×10^{-10} to 1.0×10^{-3} copies per copy 16S-rRNA, with a median of 1.2×10^{-6}, and a mean of $7.5 \times 10^{-5} \pm 2.0 \times 10^{-4}$ copies per copy 16S-rRNA for 43 samples (Table 2, Figure 10, Figure 20). Relative abundances of *tetW* were also significantly higher in Nebraska ($p=0.026$) (Figure 20).

No significant differences in either total abundance or relative proportion of *tetW* genes were observed for average, minimum, or maximum values between reference conditions (Figure 20), ecoregions (Figure 21), or hydrologic units (Figure 22).

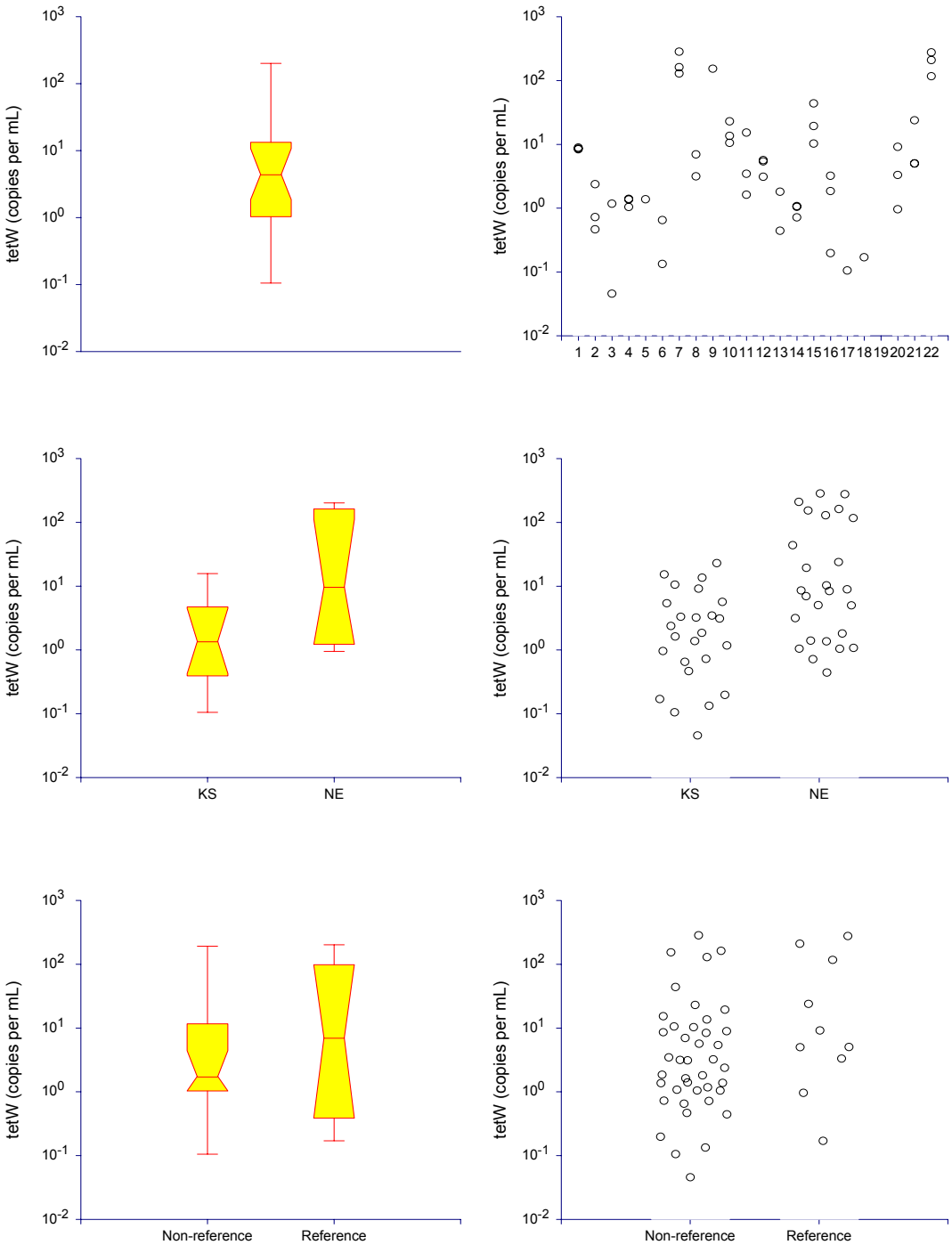

Figure 17. Summary plots for tetW gene counts by state and reference category. Box plots (a) for all measurements, (b) by state, and (c) by reference category; and dot plots (d) for all measurements, (e) by state, and (f) by reference category.

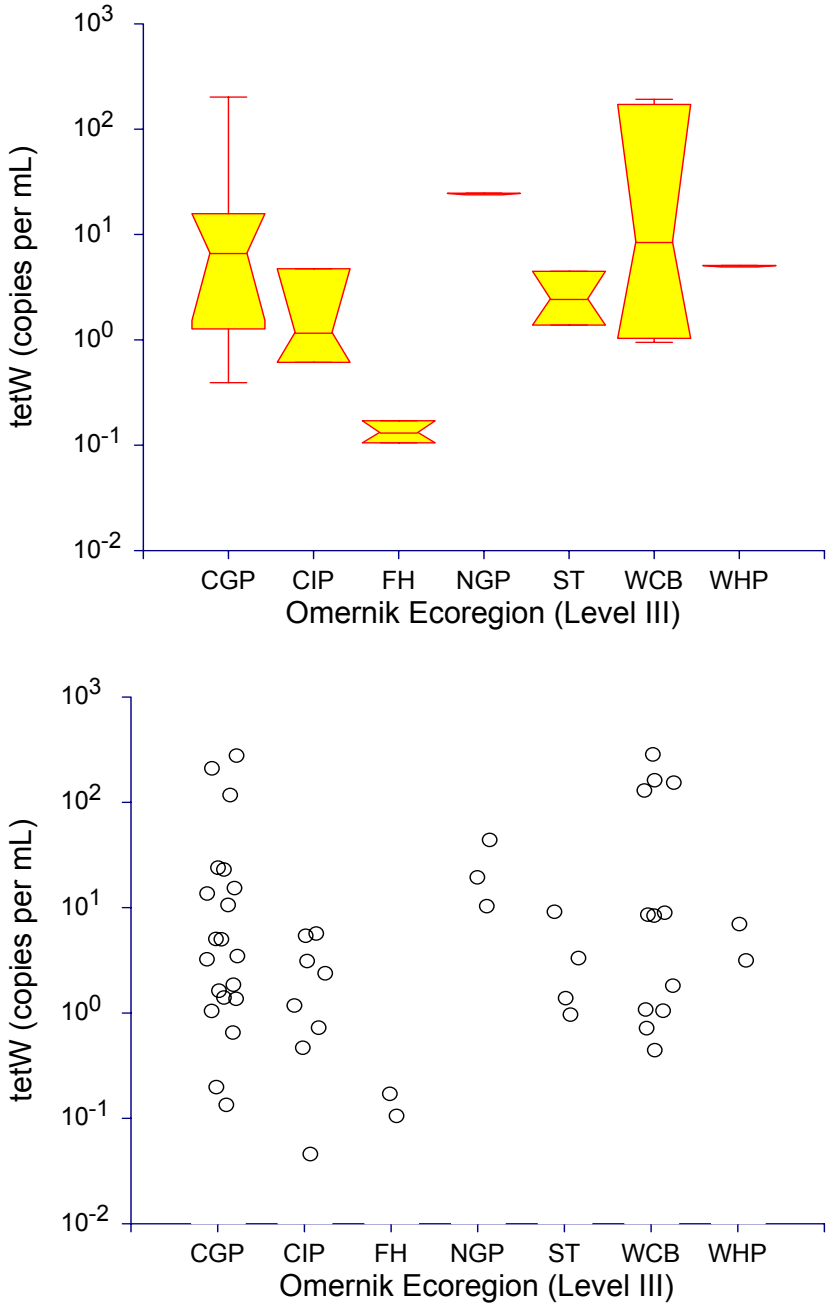

Figure 18. Box plot and dot plot of tetW gene counts by Omernik Level III Ecoregion.
NGP: Northern Great Plains; WHP: Western High Plains; NSH: Northern Sand Hills; NGL: Northern Glaciated Plains; WCB: Western Corn Belt Plains; CGP: Central Great Plains; ST: Southwest Tablelands; COT: Central Oklahoma Tablelands; CIP: Central Irregular Plains; OH: Ozark Highlands.

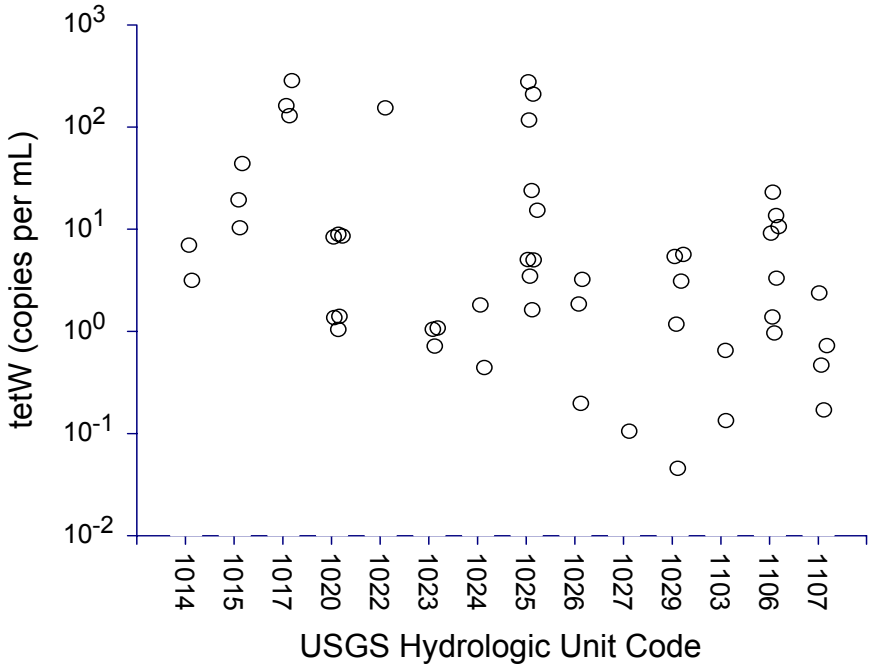

Figure 19. Dot plot of tetW gene counts by 4 digit USGS Hydrologic Unit Code.

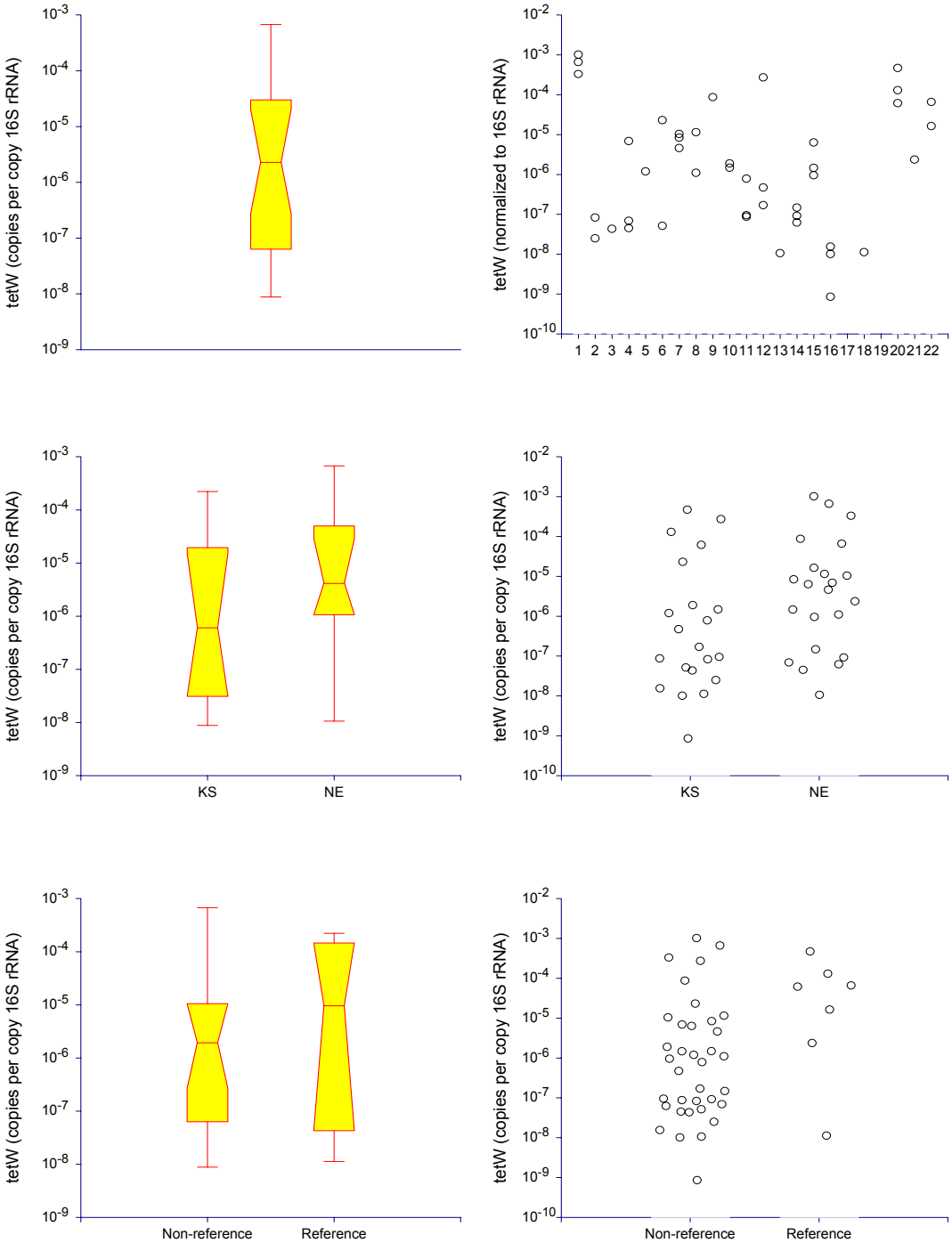

Figure 20. Summary plots for tetW gene counts relative to 16SrRNA by state and reference category.
Box plots (a) for all measurements, (b) by state, and (c) by reference category; and dot plots (d) for all measurements, (e) by state, and (f) by reference category.

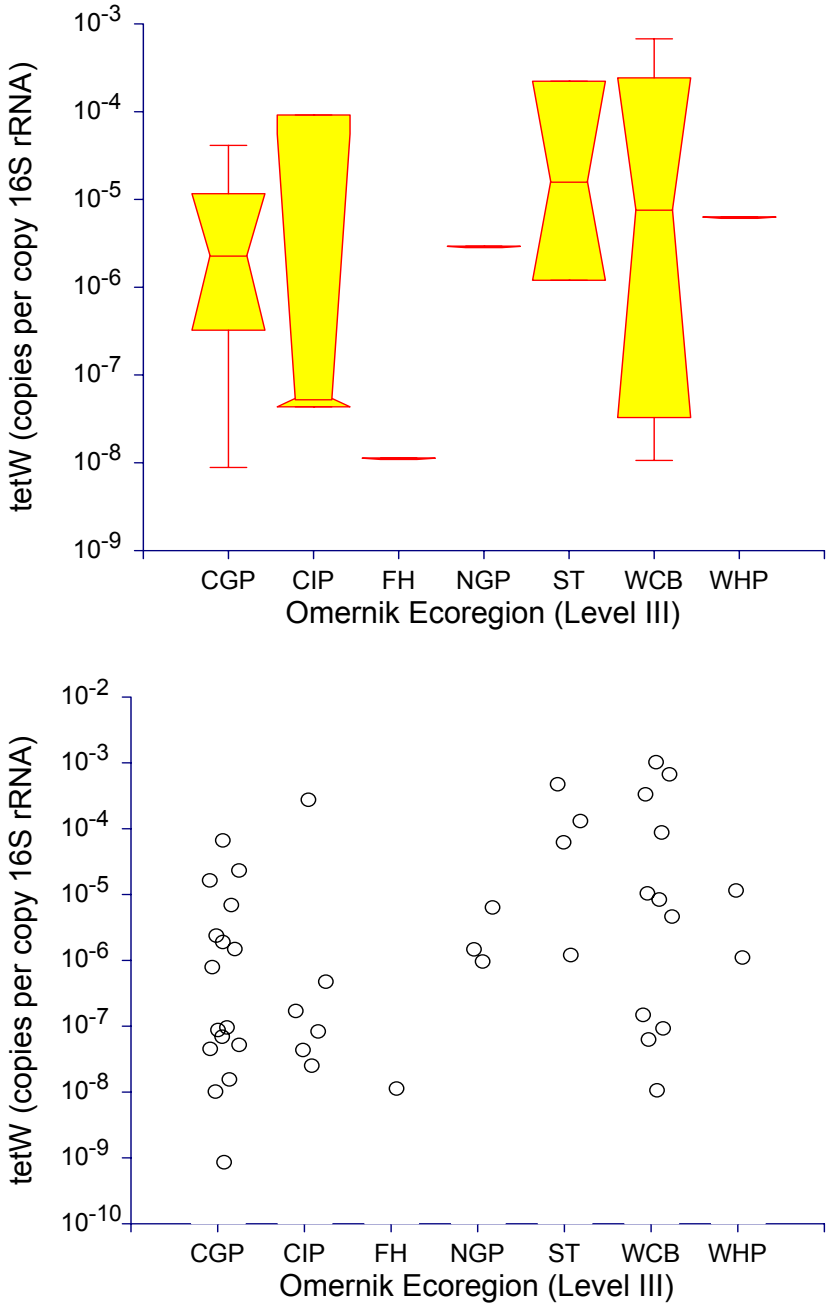

Figure 21. Box plot and dot plot of tetW gene counts relative to 16SrRNA by Omernik Level III Ecoregion.
NGP: Northern Great Plains; WHP: Western High Plains; NSH: Northern Sand Hills; NGL: Northern Glaciated Plains; WCB: Western Corn Belt Plains; CGP: Central Great Plains; ST: Southwest Tablelands; COT: Central Oklahoma Tablelands; CIP: Central Irregular Plains; OH: Ozark Highlands.

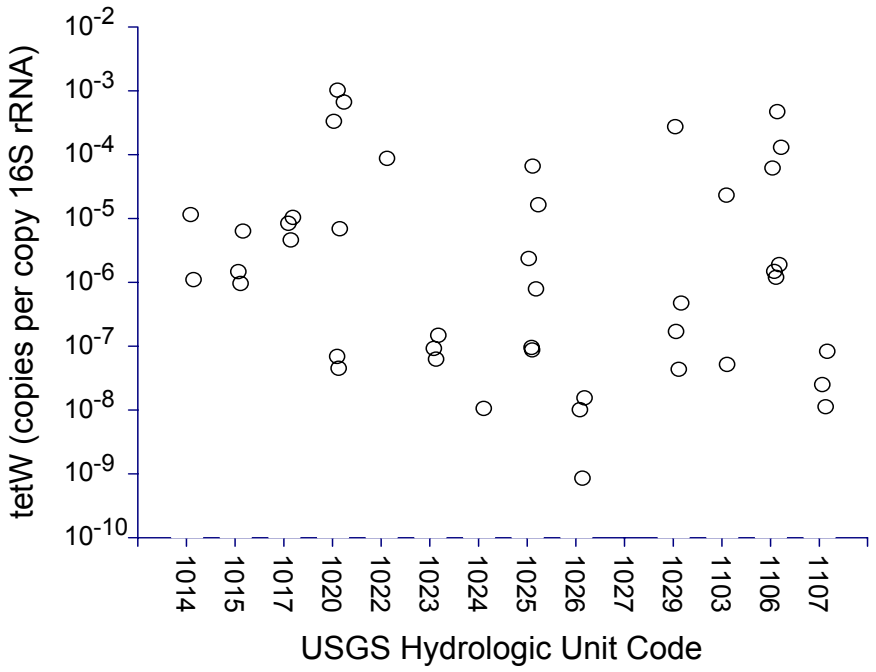

Figure 22. Dot plot of tetW gene counts relative to 16SrRNA by 4 digit USGS Hydrologic Unit Code.

tetQ

Total Abundance

Average water column tetQ ranged from 0.07 to 839 copies per mL, with a median value of 6.62 and a mean value of 69.2 ± 173 copies per mL in 51 samples (Table 2, Figure 10). Site 22 was a high outlier (839 copies per mL) compared to all other observed site averages. No significant differences between states, reference condition, ecoregion, or hydrologic unit were observed for average values, minimum values, or maximum values. Additional figures appear in Appendix D – tetQ Observed Values Figures.

Relative Proportion

Average values for water column tetQ ranged from 6.75×10^{-10} to 2.5×10^{-3} copies per copy 16S-rRNA, with a median value of 2.2×10^{-6} and a mean of $1.9 \times 10^{-4} \pm 5.3 \times 10^{-4}$ (Table 2, Figure 10). As with total abundances of tetQ, no significant differences in relative proportions of water column tetQ were observed between states, reference condition, ecoregions, or hydrologic units for average, minimum, or maximum values.

tetO

Total Abundance

The average of water column tetO ranged from 0.041 to 26.5 copies per mL, with a median value of 0.809 and a mean of 3.45 ± 6.57 copies per mL (Table 2, Figure 10). Sites 7 and 9 were high outliers both when compared to all sites and when compared by reference condition. Site 2 was a low outlier when states were compared. In addition, Site 22 was a high outlier for the Central Great Plains. Despite these outliers, no significant differences in total abundance of tetO, measured either as averages, minimums, or maximums, were observed between states, reference conditions, or ecoregions. However, hydrologic unit 1025 was significantly higher than unit 1029 when compared using maximum tetO values. Additional figures appear in Appendix E – tetO Observed Values Figures.

Relative Proportion

Average values of tetO measured as relative proportion ranged from 6×10^{-10} to 2.0×10^{-4} copies per copy 16S-rRNA, with a median value of 2.25×10^{-7} and a

mean value of $1.13 \times 10^{-5} \pm 3.36 \times 10^{-5}$ copies per copy 16S-rRNA (Table 2, Figure 10). No significant differences were observed in average, maximum, or minimum relative tetO proportions between states, reference conditions, ecoregions, or hydrologic units.

Predicted Values and Spatial Extents

Probability Sampling

Probability sampling provides a rigorous, statistically viable framework for estimating the properties of a population based on a randomly selected subset, as is often done for political or public opinion polling. This method of sampling is also widely employed by federal (USEPA) and state (KS, NE, AK, SC, MD, WV, IN) agencies to cost-effectively assess the quality of aquatic resources. The Wadeable Streams Assessment: A Collaborative Survey of the Nation's Streams (WSA) (USEPA 2006) is one such project. All of the tetracyclines and tetracycline resistance gene data presented herein were collected concurrently with the WSA to leverage its sampling efforts and underlying statistical framework for analysis.

A Venn diagram is useful to illustrate the relevant terminology of probability sampling (Figure 23). The conceptual group of all potential streams of interest – in the case of the WSA, all wadeable, perennial streams of the conterminous United States and for this study all wadeable, perennial streams of Kansas and Nebraska – is referred to as the "target population." The physical estimation of that group – the stream network identified by geographic information systems and hydrology data – is referred to as the "sampling frame." The sampling frame may miss portions of the

study population (referred to as "undercoverage"), and it may include portions outside the study population (referred to as "overcoverage"). The "sample" is a subset of the sampling frame that has been selected using a spatially balanced, probability based survey design – in the case of the WSA, a Generalized Random Tessellation Stratified Design (GRTS) (Stevens Jr. and Olsen 2003) was used. Finally, the "sampled population" is the collection of sampleable, target streams from which the sample was taken. It is the intersection of the target population and the sample frame, less those streams that are unsampleable (e.g., due to denied access, physical barriers, or other reasons). In other words, the sampled population is the physically estimated, sampleable portion of the target population. It is the population, which the sample directly approximates. The total measured or estimated linear distance of any group of streams is referred to as that group's "stream length," and the stream lengths of the sample and of the sampled population are used to generate cumulative distribution functions for parameters measured in the sample.

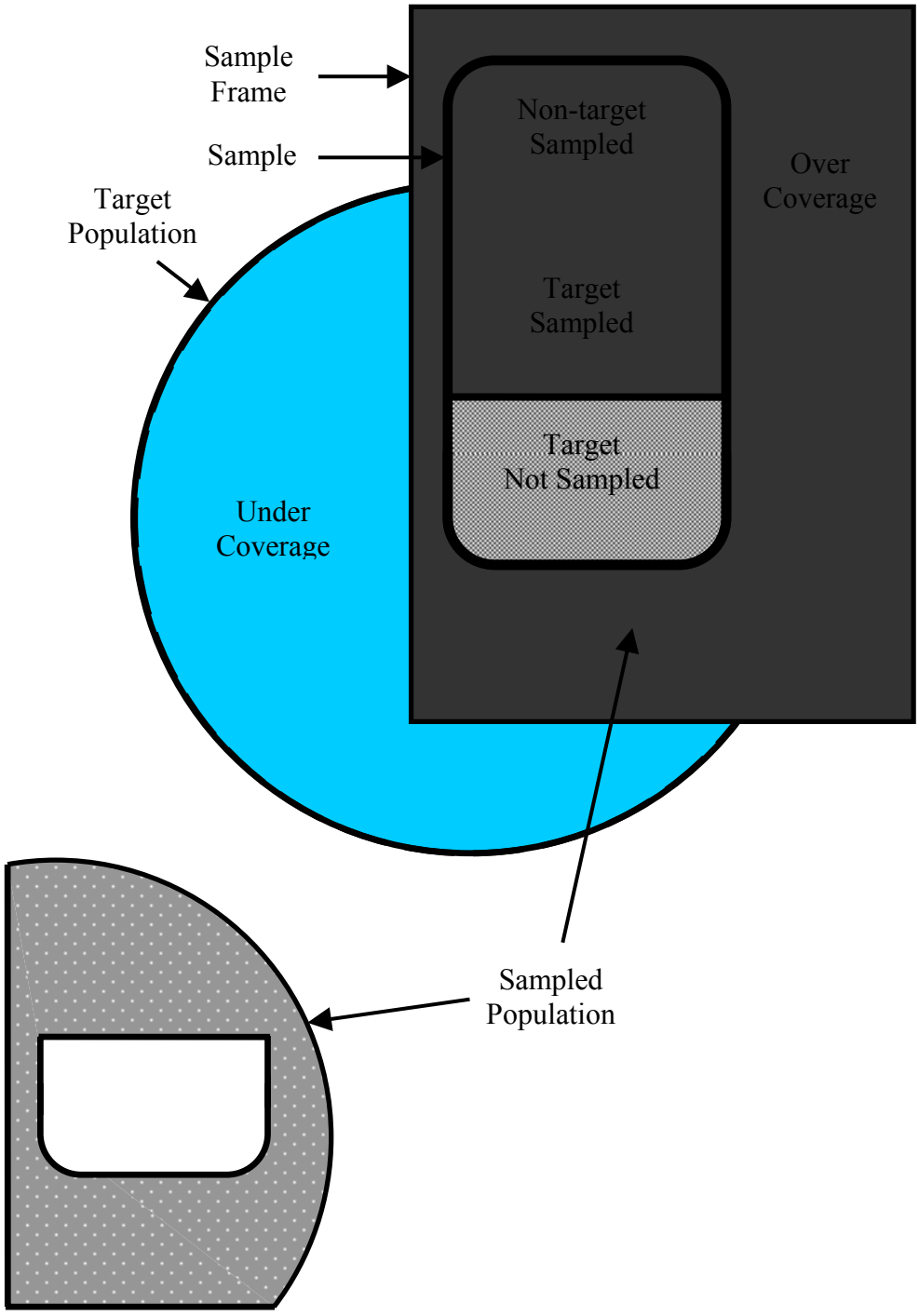

Figure 23. Venn diagram of terminology for probability sampling.
The sampled population is the intersection of the target population and the sample frame, less the target not-sampled portion. See text for full description.

Representativeness of a sample, or the potential for valid, statistical extrapolation of the sample data to the sampling frame, relies on a spatially balanced, probability based sampling algorithm. The GRTS sampling algorithm used in the WSA selected sites across two nationwide groupings to achieve spatial balance. The first grouping consisted of the 10 USEPA administrative regions (e.g., USEPA Region 7, which comprises Kansas, Nebraska, Iowa, and Missouri). The second grouping consisted of 9 WSA ecoregions, developed specifically for the WSA study (and not to be confused with Omernik ecoregions). For each USEPA region and WSA ecoregion, 50 primary sites and 150 replacement sites were randomly selected from the stream network identified within its boundaries. Some states (e.g., IA, MT, ND, SD, WY) increased the number of sites in their state to improve the precision of local analyses. All told, 1,392 sites were actually sampled as part of the WSA.

Nationwide, 1,079,952 km of combined stream length, or approximately 90% of the length of perennial streams and rivers in the conterminous US, comprised the sample frame. Kansas and Nebraska collectively belong to three WSA ecoregions (Northern Plains, Southern Plains, Temperate Plains) and one USEPA administrative region (Region 7). The WSA probability sites selected in Kansas and Nebraska were based on random tessellation of these larger regions, rather than the state boundaries themselves. After all of the statistical machinery of site selection and all of the field efforts for successful sampling, 9 sites in Kansas and 8 sites in Nebraska were sampled. These sites represent the final target sampled stream lengths: 8,290 km (26% of the sample frame) for Kansas, 15,554 km (59% of sample frame) for

Nebraska, and 23,844 km (41% of sample frame) for both states combined. The sample frame lengths were 31,566 km, 26,223km, and 57,789km, respectively for Kansas, Nebraska, and both states combined.

Predicted Values and Spatial Extents from this Study

Based on the determined sample stream and frame lengths for Kansas and Nebraska, the observed data from this study were used to calculate cumulative distribution functions (cdf's) for both estimates and confidence intervals of tetracyclines, of total gene counts, and of resistance genes counts. Calculation and graphing of cdf's were performed via the spsurvey package for R 2.4.1 developed by Kincaid et al. (2008). While the estimates provided by these cdf's apply directly to the sampled population, extrapolation of the estimates to the target portion of the sampling frame can only be made correctly if the target non-sampled sites (i.e., sites not sampled due to denied access, physical barriers, and other reasons) occurred both randomly and independent of site characteristics. For example, if access to sites with high tetracycline values were systematically denied, then any extrapolation would be skewed toward lower levels. Based on the information available, however, denials appeared to be both independent of site characteristics (at least in terms of watershed characteristics and stream size) and randomly occurring. Further, for the estimates to apply to the target population as a whole (i.e., all perennial, wadeable streams in Kansas and Nebraska), not only must both previous assumptions hold (i.e., random non-sampling and independence of site characteristics from denial), but also the undercoverage areas of the target population must share the same properties as the

sampled population. Additional random sampling would be necessary to verify this assumption.

The following quantifiable estimates of ambient tetracycline and tetracycline resistance genes are for the thousands of kilometers represented by the sampled population of this study (see Appendix F – Predicted Values Tables for tabular versions of the figures included in the main text). These cumulative distributions also provide a reasonable first estimate for tetracycline and tetracycline resistance genes in all wadeable, perennial stream kilometers in Kansas and Nebraska, though additional sampling is necessary for verification. For most analytes, there were no significant differences between levels observed in Kansas and those observed in Nebraska. Therefore, cdf's for these compounds are presented inclusive of stream lengths in both states. For *tetW*, statistical differences were observed between Kansas and Nebraska, and a cdf for each state is therefore provided. Finally, it should also be noted that these estimates are likely conservative, since the values reflect observations of ambient water column concentrations, which are known to be potentially significantly lower than biofilm, soil, and sediment concentrations. Nonetheless, conservative values of ambient concentrations should provide a quantifiable lower threshold for these analytes in the environment.

Tetracyclines

Using spatial prediction methods developed for the National Wadeable Streams Assessment GRTS probability design (Stevens Jr. and Olsen 2003), 50% (or approximately 11,900) of the stream kilometers in Kansas and Nebraska are projected

to have 245 ppt or less of tetracyclines with a 95% confidence range of the estimated mean between 182 and 284 ppt (Figure 24).

Total Numbers of Genes

Average 16S-rRNA counts for 50% of Kansas and Nebraska stream kilometers (approximately 11,900 km) are predicted to be 9.97 x 106 with a 95% confidence range of the estimate between 2.14 x 10^6 and 1.91 x 10^7 (Figure 25). Maximum and minimum 16S-rRNA counts are predicted to be within one order of magnitude of these estimates.

Resistance Genes

tetW

Since observed tetW levels are significantly different between Kansas and Nebraska, separate predictions are generated for each state. In Kansas, average tetW counts in 50% of stream kilometers (approximately 4,150 km) are predicted to be 1.30 copies per mL with a 95% confidence range of 0.439 to 4.97 copies per mL as total abundance (Figure 26, see Appendix H). The average relative proportion of tetW for 50% of Kansas stream kilometers was predicted to be 8.68 x10-7 copies per copy 16S-rRNA with a range of 4.23x10^{-8} to 1.73x10^{-5} copies per copy 16S-rRNA (Figure 30, see Appendix H). In Nebraska, average tetW counts in 50% of stream kilometers (approximately 7,750 km) are predicted to be 4.27 copies per mL with a 95% confidence range of 0.960 to 101 copies per mL as total abundance (Figure 27, see Appendix H). The average relative proportion of tetW for 50% of Nebraska stream kilometers was predicted to be 3.22 x 10^{-6} copies per copy 16S-rRNA with a

95% confidence range of 2.58 x 10^{-8} to 6.96 x 10^{-5} copies per copy 16S-rRNA (Figure 31, see Appendix H).

As with total gene counts, both the predicted maximum values and the predicted minimum values for tetW are within one order of magnitude of the predicted average values for both Kansas and Nebraska, in terms of both total abundance and relative proportion.

tetQ

Average tetQ counts in 50% of stream kilometers of Kansas and Nebraska (approximately 11,900 km) are predicted to be 5.26 copies per mL with a 95% confidence range of 1.99 to 14.9 copies per mL as total abundance (Figure 28, see Appendix F) and 4.77 x 10^{-6} copies per copy 16S-rRNA with a 95% confidence range of 2.01 x 10^{-7} to 2.78 x 10^{-5} copies per copy 16S-rRNA as a relative proportion (Figure 32, see Appendix F). Maximum values as both total abundance and relative proportion are predicted to be within the same order of magnitude as average values, while minimum values are predicted to be the same order of magnitude for total abundance, but one order of magnitude less as a relative proportion.

tetO

Average tetO counts in 50% of stream kilometers of Kansas and Nebraska (approximately 11,900 km) are predicted to be 0.609 copies per mL with a 95% confidence range of 0.158 to 1.48 copies per mL as total abundance (Figure 29, see Appendix F), and 4.29 x 10^{-7} copies per copy 16S-rRNA with a 95% confidence range of 1.16 x 10^{-8} to 3.40 x 10^{-6} copies per copy 16S-rRNA as a relative proportion

(Figure 33, see Appendix F). As with tetQ, maximum values as both total abundance and relative proportion are predicted to be within the same order of magnitude as average values, while minimum values are predicted to be the same order of magnitude for total abundance, but one order of magnitude less as a relative proportion.

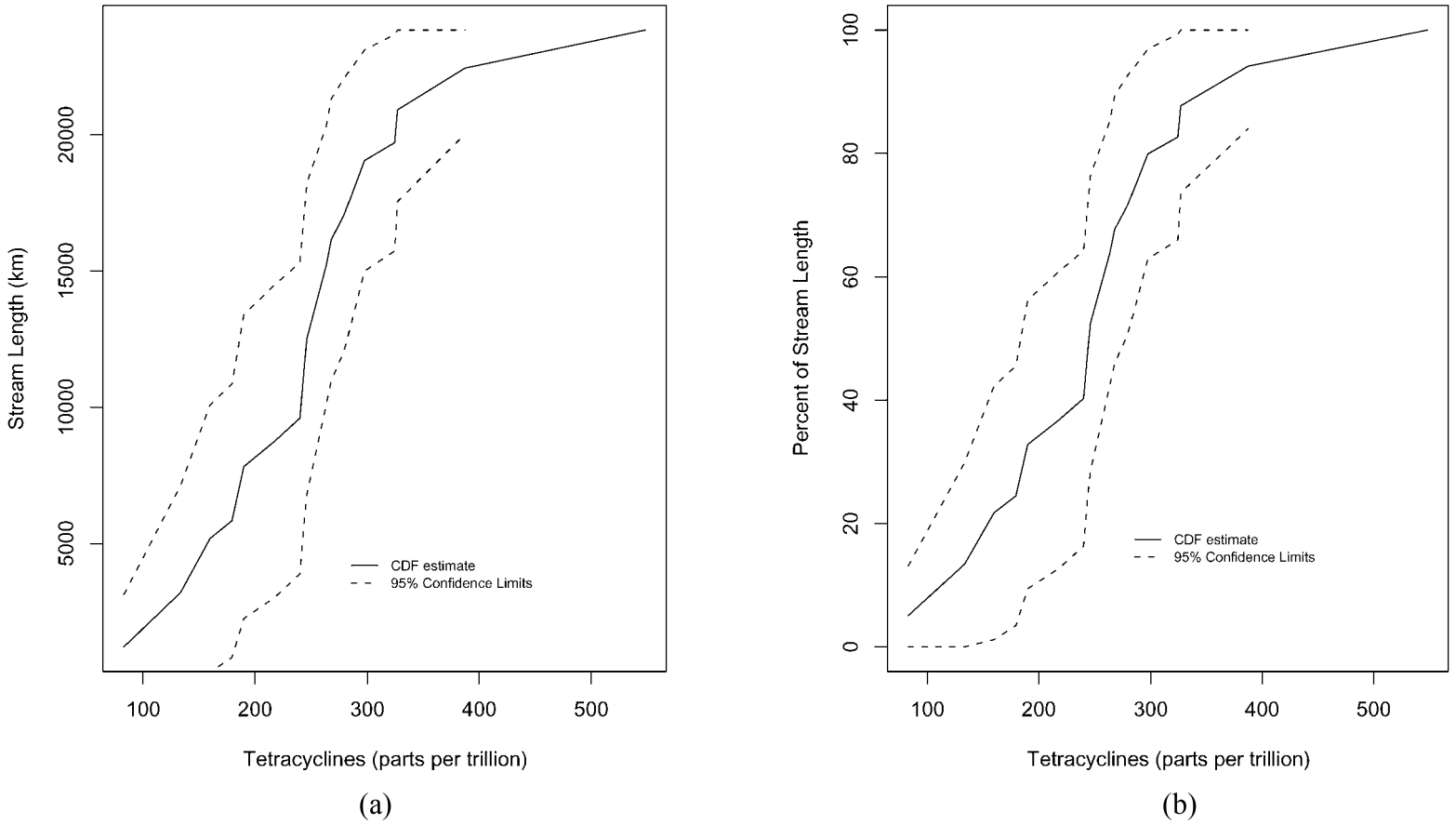

Figure 24. Cumulative distribution function by (a) stream length and (b) percent of stream length for predicted concentration of tetracyclines in perennial, wadeable streams of Kansas and Nebraska.

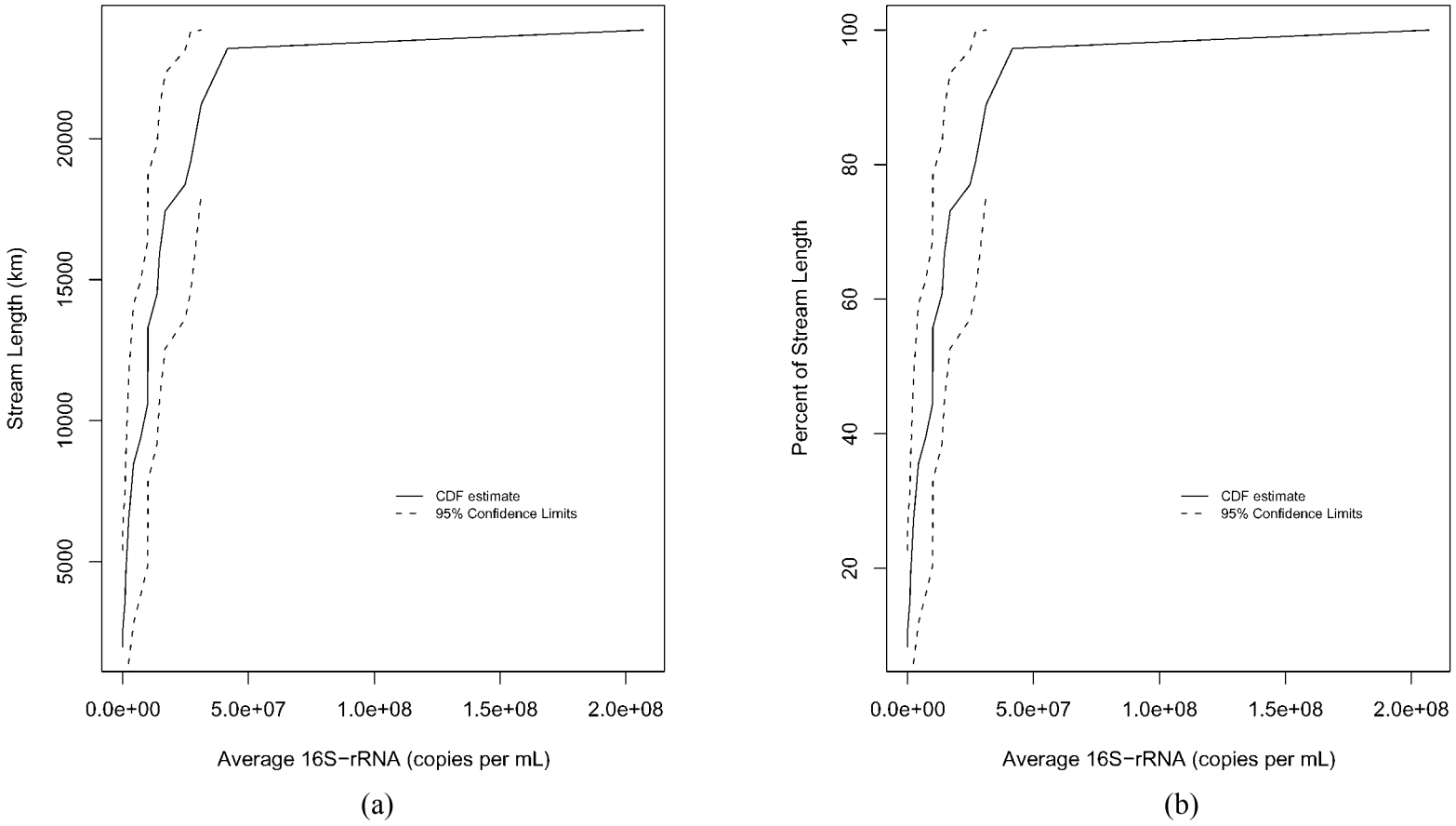

Figure 25. Cumulative distribution function by (a) stream length and (b) percent of stream length for the predicted average of 16SrRNA concentrations in perennial, wadeable streams of Kansas and Nebraska.

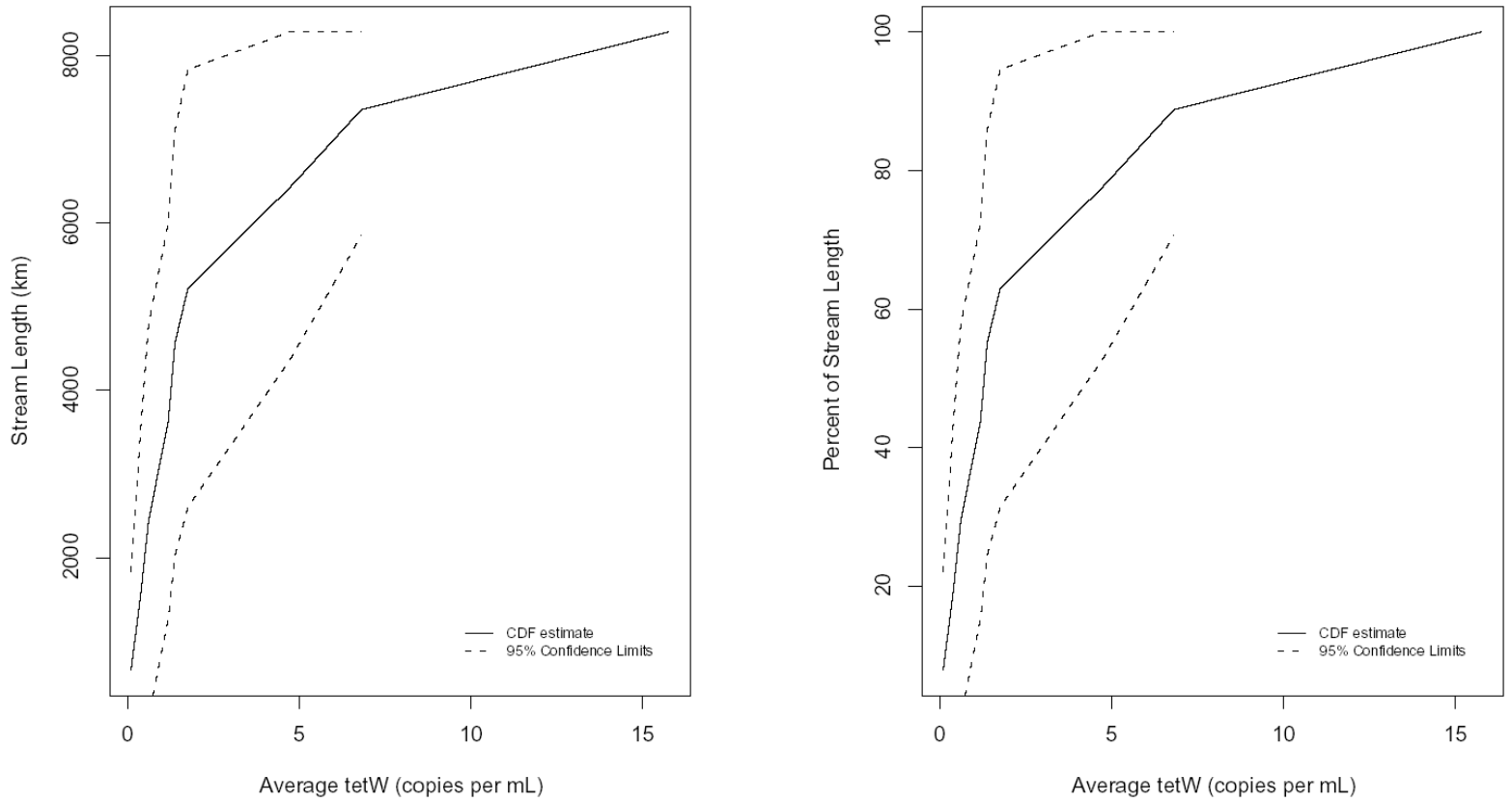

Figure 26. Cumulative distribution function by (a) stream length and (b) percent of stream length for the predicted average of tetW concentrations in perennial, wadeable streams of Kansas alone.

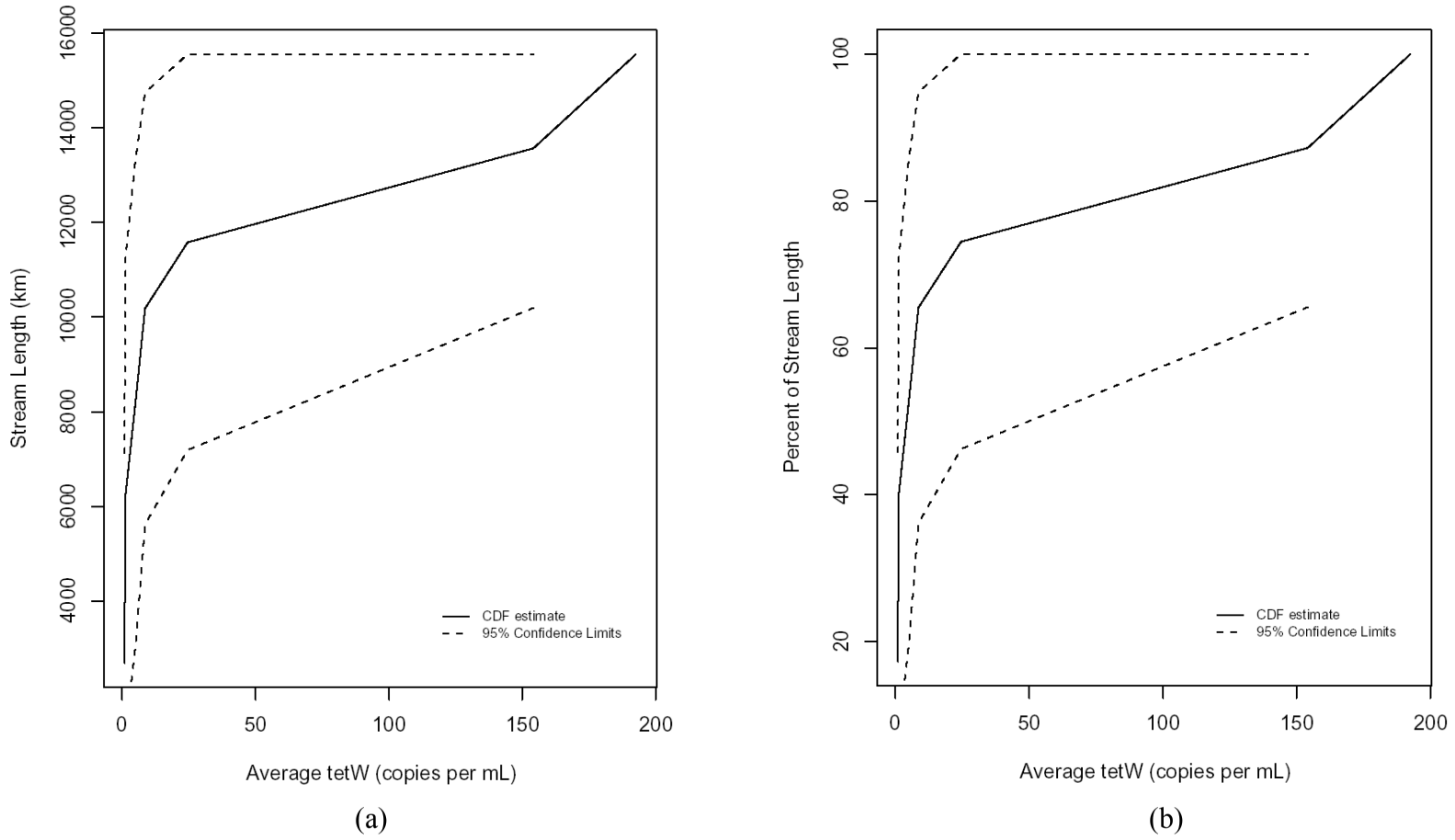

Figure 27. Cumulative distribution function by (a) stream length and (b) percent of stream length for the predicted average of tetW concentrations in perennial, wadeable streams of Nebraska alone.

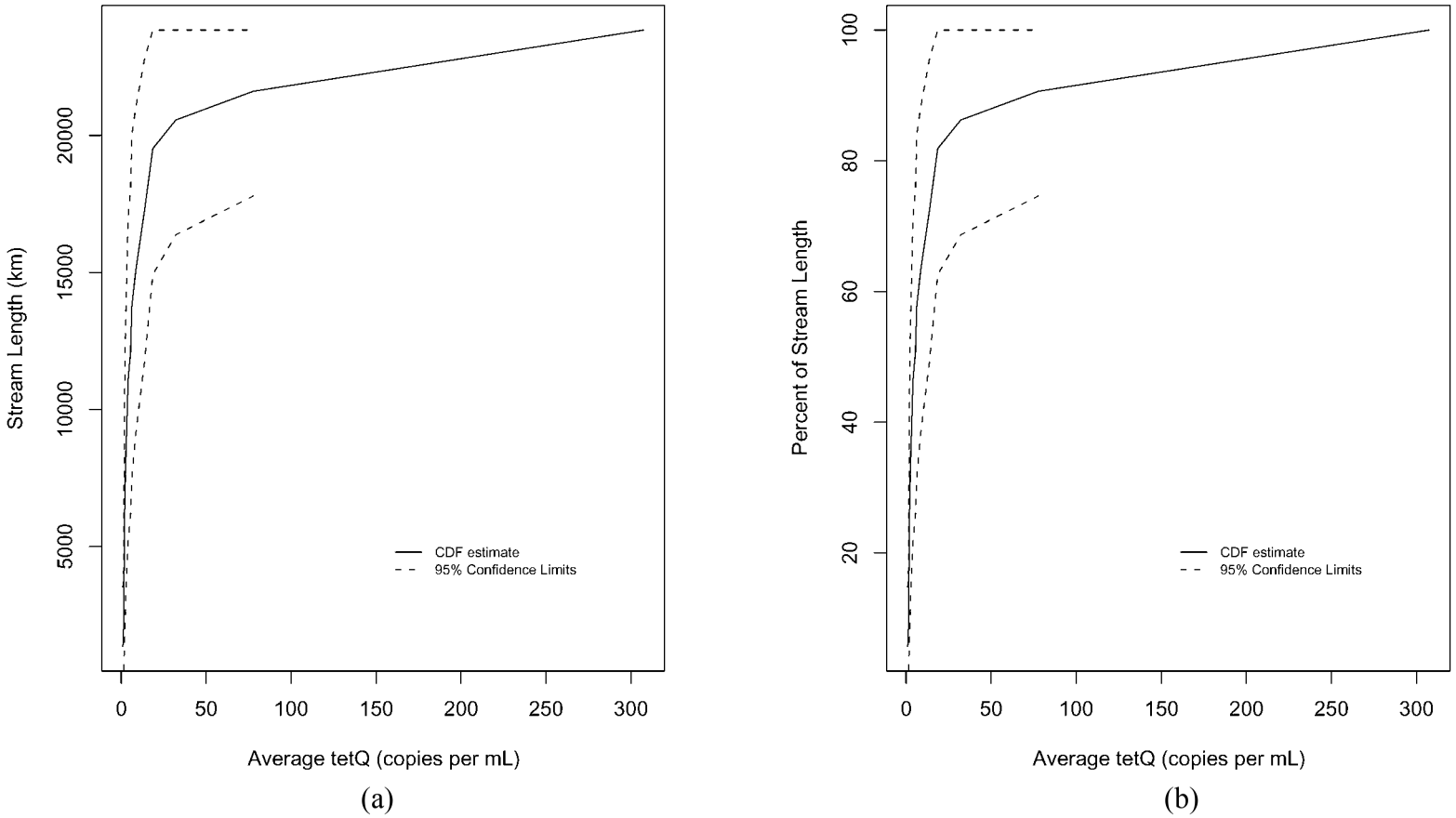

Figure 28. Cumulative distribution function by (a) stream length and (b) percent of stream length for the predicted average of tetQ concentrations in perennial, wadeable streams of Kansas and Nebraska.

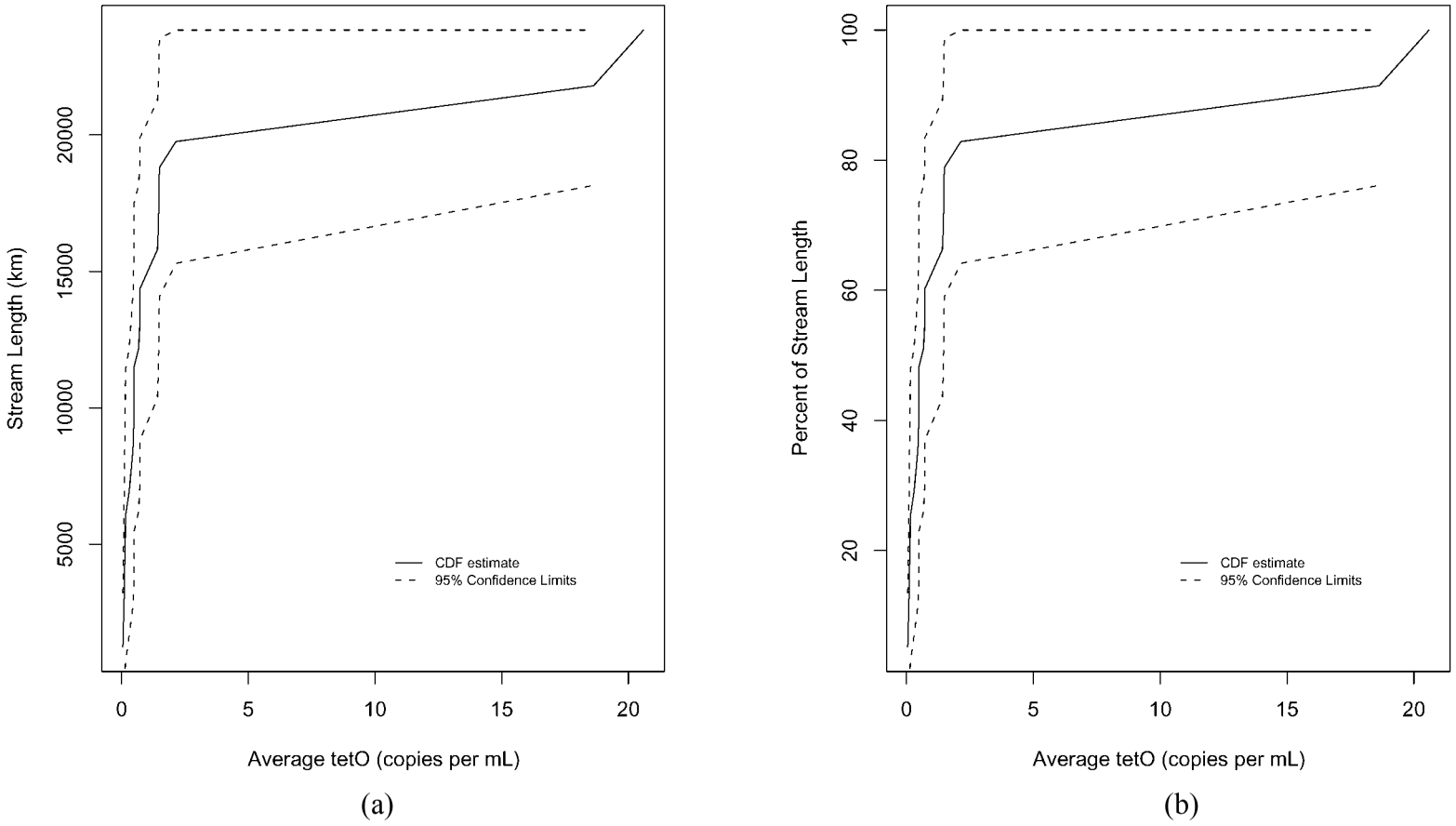

Figure 29. Cumulative distribution function by (a) stream length and (b) percent of stream length for the predicted average of tetO concentrations in perennial, wadeable streams of Kansas and Nebraska.

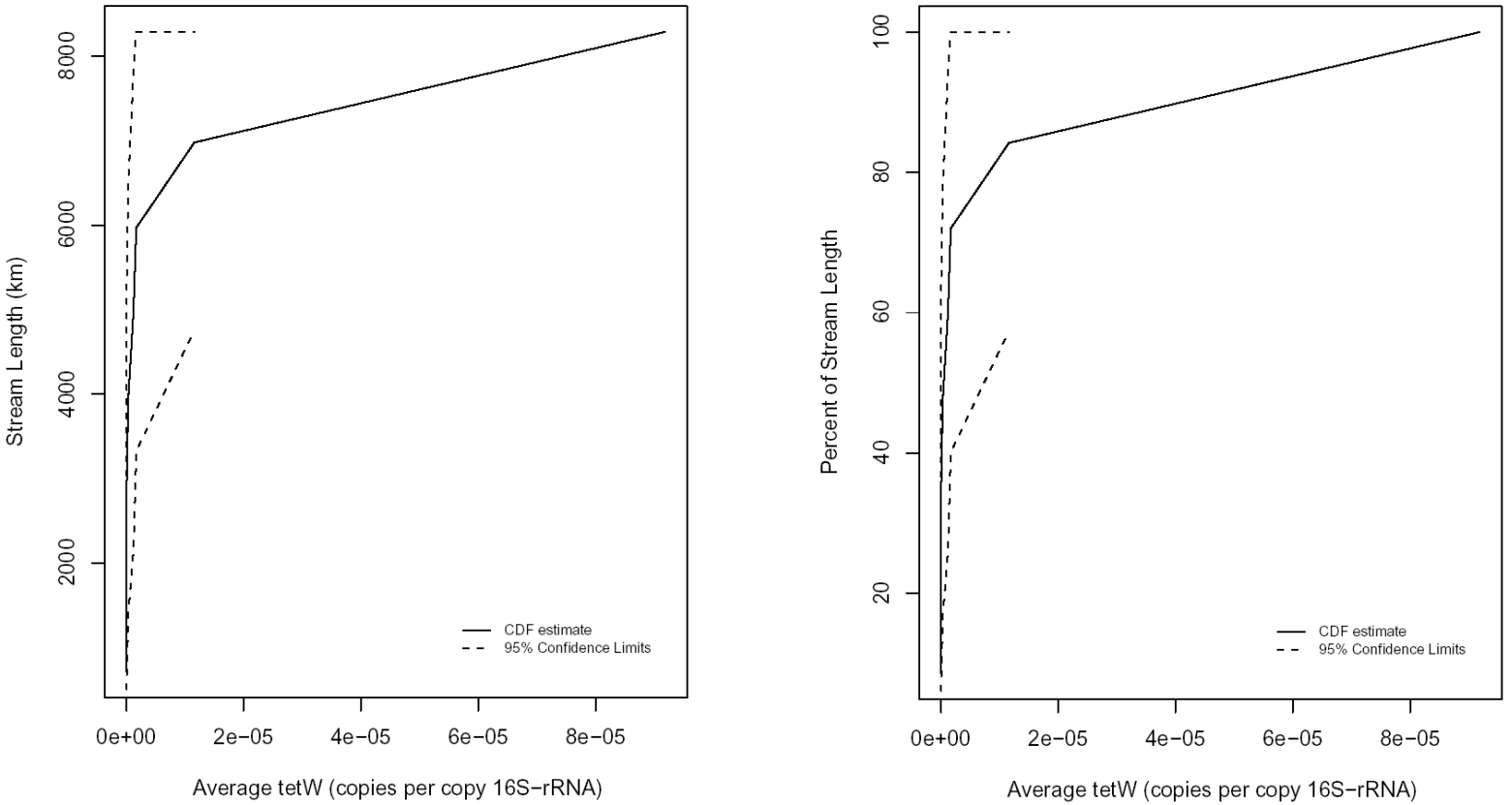

Figure 30. Cumulative distribution function by (a) stream length and (b) percent of stream length for the predicted average of tetW concentrations relative to 16SrRNA in perennial, wadeable streams of Kansas alone.

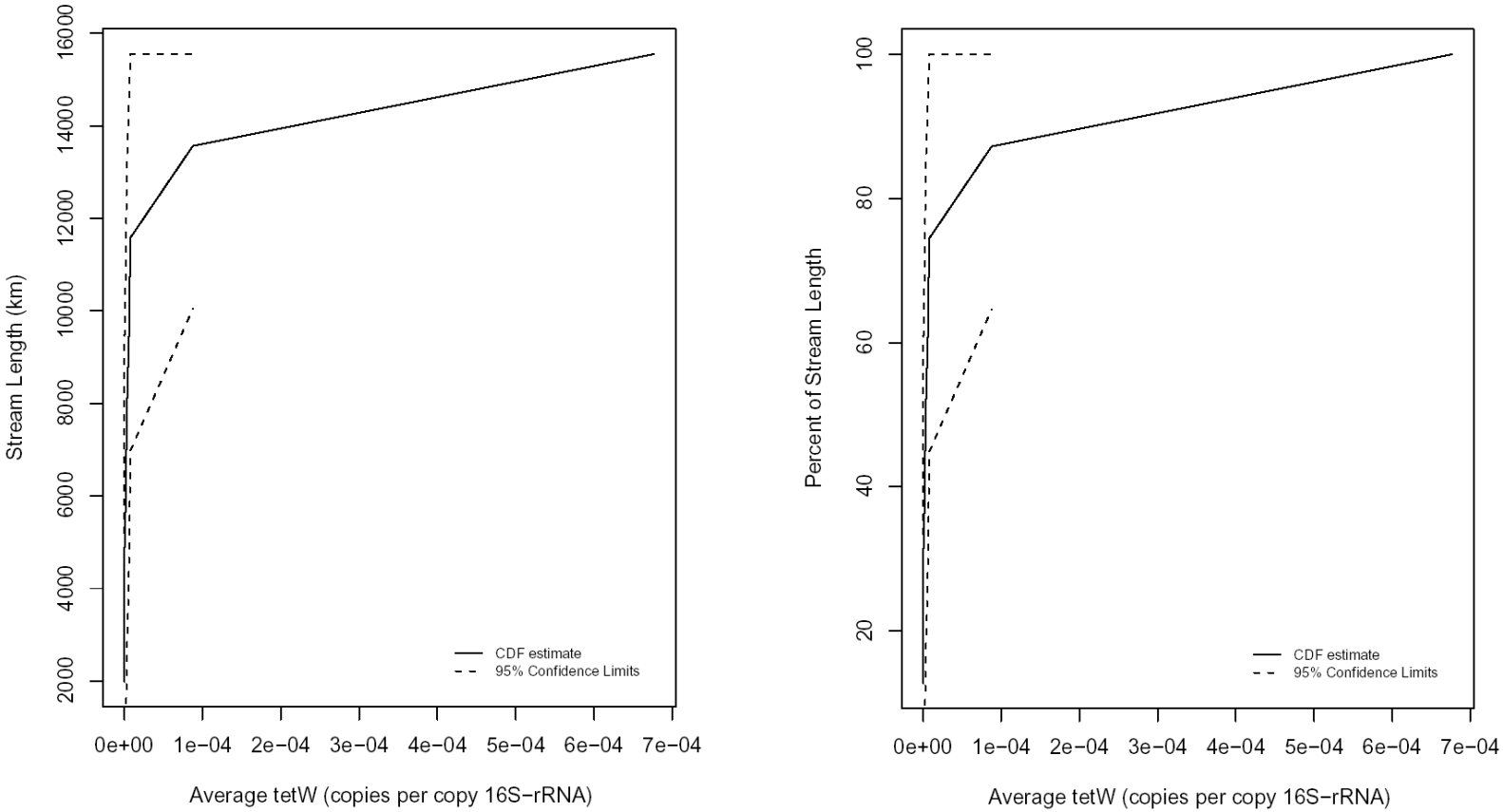

Figure 31. Cumulative distribution function by (a) stream length and (b) percent of stream length for the predicted average of tetW concentrations relative to 16SrRNA in perennial, wadeable streams of Nebraska alone.

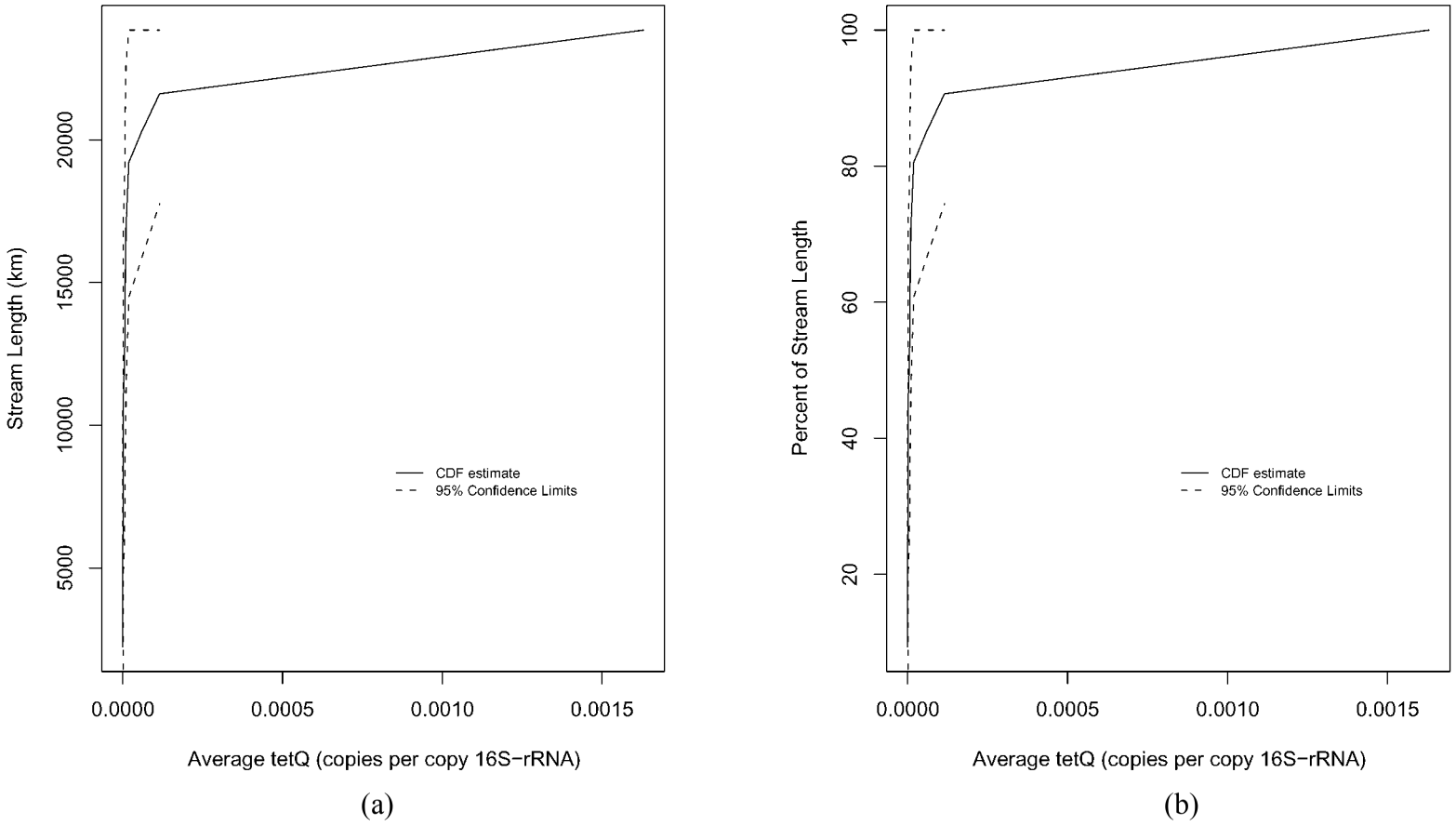

Figure 32. Cumulative distribution function by (a) stream length and (b) percent of stream length for the predicted average of tetQ concentrations relative to 16SrRNA in perennial, wadeable streams of Kansas and Nebraska.

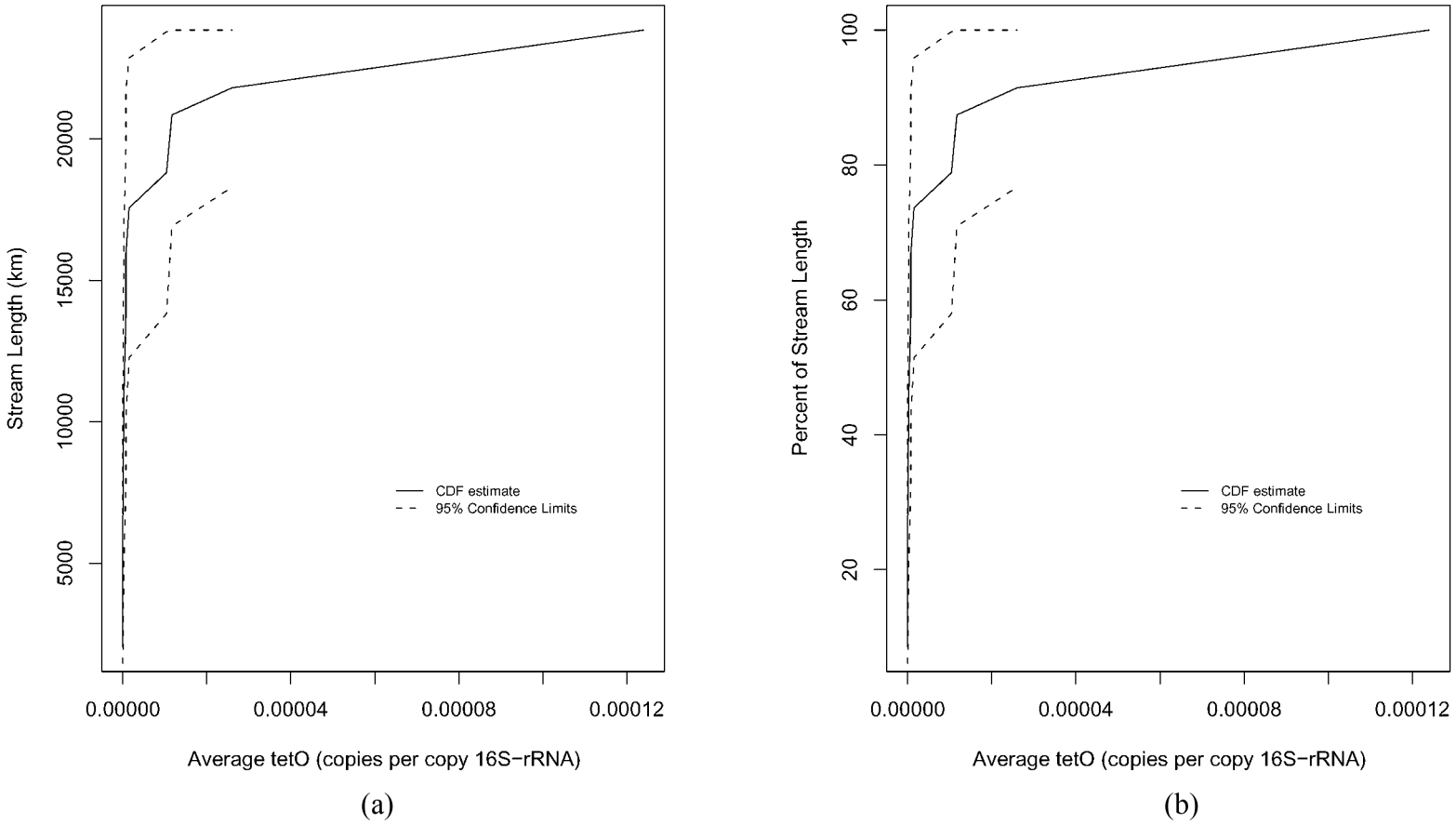

Figure 33. Cumulative distribution function by (a) stream length and (b) percent of stream length for the predicted average of tetO concentrations relative to 16SrRNA in perennial, wadeable streams of Kansas and Nebraska.

Q2: Are Tetracyclines, Total Gene Counts, and tet Resistance Genes Related in the Environment?

The second goal of this study is to examine the relationship among tetracyclines, total gene counts, and *tet* gene levels in the perennial, wadeable streams of Kansas and Nebraska. Previous studies have shown inconsistent results regarding the co-occurrence of tetracyclines and tetracycline resistance genes in the environment. When tetracyclines are sufficiently high in concentration, non-resistant organisms are killed, and tetracyclines inversely correlate with total gene counts. Similarly, in the presence of high concentrations of antibiotics, antibiotic resistant organisms are often the most numerous (or only) organisms present, which yields a positive correlation between tetracyclines and tetracycline resistance gene counts in these situations (Smith et al. 2004b). However, the relatively short half-life of tetracyclines in surface waters (Chopra and Roberts 2001; Doi and Stoskopf 2000; Kuhne et al. 2000; Loftin et al. 2008; Nelson 2001; Qiang and Adams 2004) suggests that the presence of tetracyclines in environmentally active concentrations is an unlikely circumstance, unless consistent sources of tetracyclines are ubiquitous and at high concentration. In fact, resistance genes have been observed in the absence of significant levels of tetracyclines (Engemann et al. 2006; Peak et al. 2007; Pei et al. 2006). Moreover, tetracycline resistance genes are known to be conserved in some genera, either through selection by other stressors (e.g. concurrent conference of resistance to metals), by inclusion in the genome of organisms that naturally produce tetracyclines, by horizontal transfer from environmental gene reservoirs, or by

mediated evolutionary costs (i.e., reduced evolutionary pressure to lose the gene) provided by a combination of altered selection, natural occurrence, and horizontal transfer. Therefore, the *a priori* expectation of this study was that resistance genes would occur regardless of observed tetracycline levels, but would occur at low levels in the ambient environment. Where tetracyclines were observed to be high, then resistance genes were also expected to be high, though this case was taken to be uncommon given the likelihood that tetracyclines would be low under environmental conditions.

As observed, the ambient tetracyclines did not significantly correlate with total gene counts, nor did they correlate with *tet* genes, either as total abundances or as relative proportions (Figure 10, Table 4). However, tet genes were significantly and highly correlated to each other, both in total abundance and relative proportion (Figure 10, Table 4). Significant correlations between total gene counts and relative proportions of *tet* genes also occurred trivially as an artifact of their calculation (relative proportion of *tet* genes = total abundance of *tet* genes / total gene count) (Figure 10, Table 4). Though Smith et al. (2004b) found correlation between *in situ* tetracyclines and total abundance of resistance genes, both Engemann et al. (2006) and Peak et al. (2007) found no statistical correlation between tetracycline levels and resistance genes in swine lagoons, as was observed in this study. To the author's knowledge, no significant statistical correlations of occurrence have been previously reported among different *tet* genes in general, and among *tetW*, *tetQ*, and *tetO* in particular.

In a recent review, Roberts (2005) noted that *tetW* and *tetO* are among both the most widely found and most commonly transferred tetracycline resistance genes. In fact, *tetW* has the third largest documented host range of all *tet* and *otr* genes, spanning Gram-positive, Gram-negative, aerobic, and anaerobic bacteria (Roberts 2005). Despite the relative ubiquity and homology of these two genes, however, their transfer mechanisms are not entirely the same. *Tet*O genes are usually associated with conjugative plasmids, while *tetW* genes are normally associated with conjugative transposons, which are much more prolific. Transfer mechanisms for *tetQ* are less well documented. All three genes do code for ribosomal protection, but since these genes reside on different mobile genetic elements, it is perhaps a bit surprising to find high correlation in environmental samples. Moreover, such high correlations could suggest some mechanism for transfer of these genes in connection with one another if the genes are contained within the same organisms *in vivo*.

Some recent evidence suggests partitioning of microbial resistance genes into biofilms (Engemann et al. 2008), and that this partitioning occurs at different rates with different genes. Since these three genes (*tetW*, *tetQ*, and *tetO*) are among the most mobile of observed genes, it is possible that the high correlation of these genes has to do with their persistence in and transfer from local biofilms.

Table 4. Pearson correlation matrix for tetracyclines (TCs) and genes. Significant correlations are indicated by bold typeface and either one ($p < 0.05$) or two asterisks ($p < 0.001$). Each correlation was calculated using 18 observations of all variables.

			log [Genes (copies per mL)]				*log [Genes (copies per copy 16S-rRNA)]*		
		TCs	*16S-rRNA*	*tetW*	*tetQ*	*tetO*	*tetW*	*tetQ*	*tetO*
	TCs	1	0.322	-0.137	-0.284	-0.101	-0.180	-0.261	-0.152
log [Genes (copies per mL)]	*16S-rRNA*		1	-0.009	-0.115	-0.074	**-0.751****	**-0.757****	**-0.765****
	tetW			1	**0.917****	**0.872****	0.462	0.382	0.312
	tetQ				1	**0.893****	0.501	0.512	0.384
	tetO					1	**0.536***	**0.523***	**0.481***
log [Genes (copies per copy 16S-rRNA)]	*tetW*						1	**0.962****	**0.970****
	tetQ							1	**0.967****
	tetO								1

Discussion

Context for Observed and Predicted Values

While the enumeration of ambient values of tetracyclines and resistance genes is valuable in itself, the raw numbers provide little insight into the overall relative condition or potential contamination of a given site in particular. Therefore, comparison with previously characterized, targeted-study sites can provide context and help to determine if the magnitude of the baseline is reasonable. Fortunately, some data are available in the literature. Recently published data were grouped into four disturbance categories: (HI) "Heavily Impacted" – by urban areas and/or agriculture, such as sewage treatment effluents, feedlot waste lagoons, feedlot soils, raw manure, and areas noted as both urban and agricultural by the authors; (LU) "Lightly Impacted Urban" – urban areas other than sewage treatment outfalls; (LA) "Lightly Impacted Agriculture" – areas such as pasture land, crop land, streams and rivers in croplands or pastures, irrigation ditches, and any sites with otherwise unidentifiable disturbance conditions; and (P) "Pristine" – areas specifically identified by the studies' authors as pristine or reference condition sites. Sites from this study are labeled as disturbance category (X) "Experimental" for comparison Appendix G – Contextual Values Of Tetracyclines (Raw Data.

Tetracyclines

Based on data from five recent targeted studies (Campagnolo et al. 2002; Hirsch et al. 1999; Kolpin et al. 2002; Mackie et al. 2006; Pei et al. 2006), the

ambient levels of tetracyclines observed in this study are relatively low compared to anthropogenically disturbed sites for both total tetracyclines (Figure 34) and various individual tetracycline compounds (Figure 35). The observed ambient values are in the range of the lower third of both heavily disturbed and lightly disturbed agricultural sites, and most are below detection limits for one study (see Appendix G – Contextual Values Of Tetracyclines (Raw Data)). Based on analysis of variance, the observed ambient levels of tetracyclines are significantly lower ($p<0.001$) than those of the heavily impacted and lightly impacted urban sites, but not significantly different than the lightly impacted agriculture or pristine sites. Still, the observed ambient tetracycline levels found in this study were also higher than those found in sites designated as "pristine" in previous studies. For completeness and additional verification, the pristine sites were also significantly lower ($p<0.001$) than the three impacted groups (HI, LU, LA).

As a further point of reference, minimum selective levels for tetracycline have been identified both *in vitro* (0.2 μg/L) and *in vivo* (6.5 to 12.8 μg/L) in gnotobiotic mice (Corpet et al. 1989). These levels correspond to 0.2 parts per million (2×10^5 ppt) and 6.5 to 12.8 parts per million (6.5×10^6 to 12.8×10^6 ppt), respectively, which are three to five orders of magnitude higher than tetracycline levels observed in this study. Further, Bahl et al. (2004) have found that *in vivo* concentrations (i.e., bioavailable in the intestinal tract) of tetracyclines in mice were 0.4% of the *in vitro* intake concentrations. In other words, the levels of tetracyclines that actually occur in the gut of animals that ingest them are about 4/1000 as high as the levels of

tetracyclines in the material the mice ingest, suggesting that the ambient tetracycline levels observed in this study pose little to no threat for direct tetracycline selection of antibiotic resistant enteric organisms. Partitioning of tetracyclines into environmental compartments that favor longevity of the compound (e.g., cool, dark soils or biofilms) may provide some additional risk, but any exposure to surface waters or high temperatures would likely degrade the compounds rapidly (i.e., with several hours).

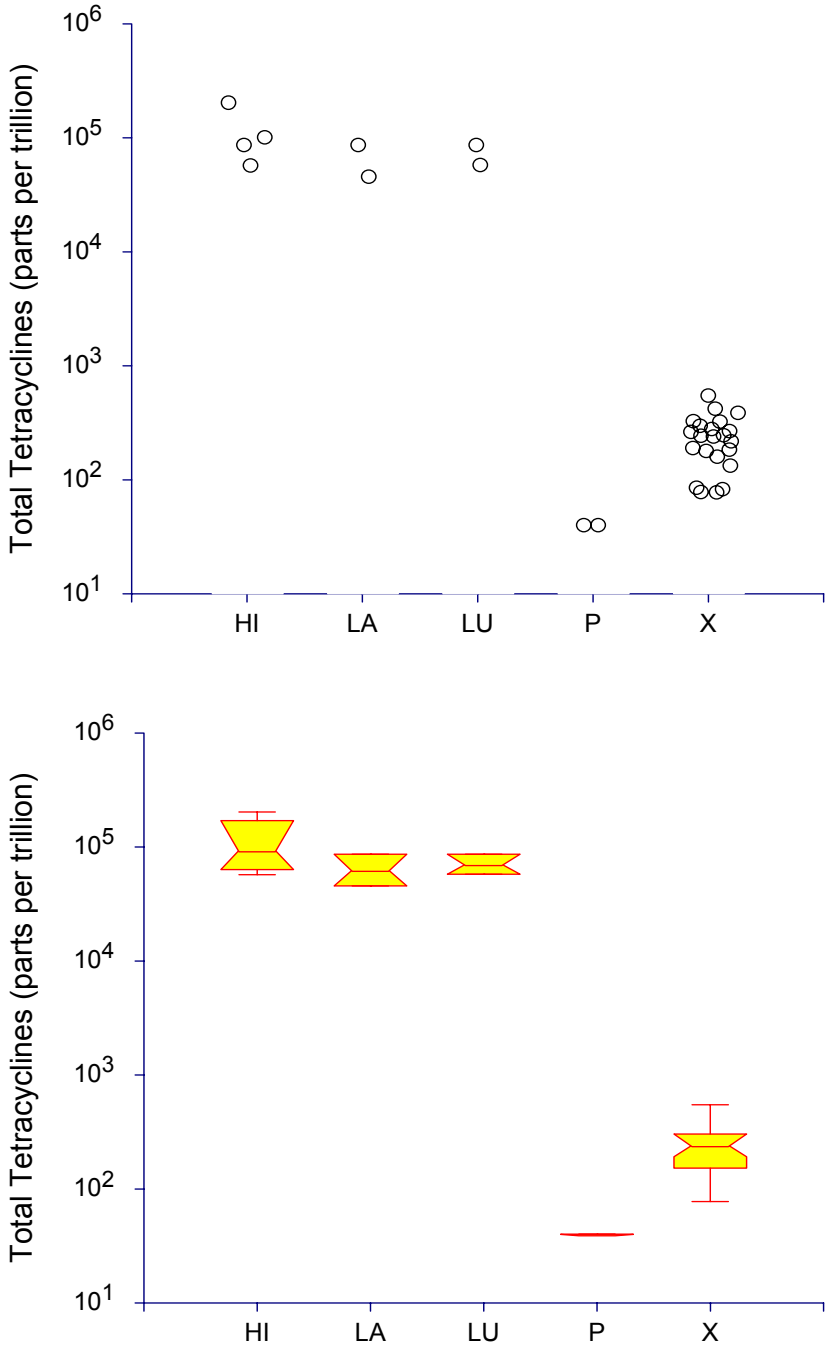

Figure 34. Contextual measurements of total tetracyclines.
Disturbance categories are (HI) heavy agriculture or heavy agriculture and urban, (LA) light agriculture or indeterminate location, (LU) urban, (P) pristine, and (X) experimental data from this survey.

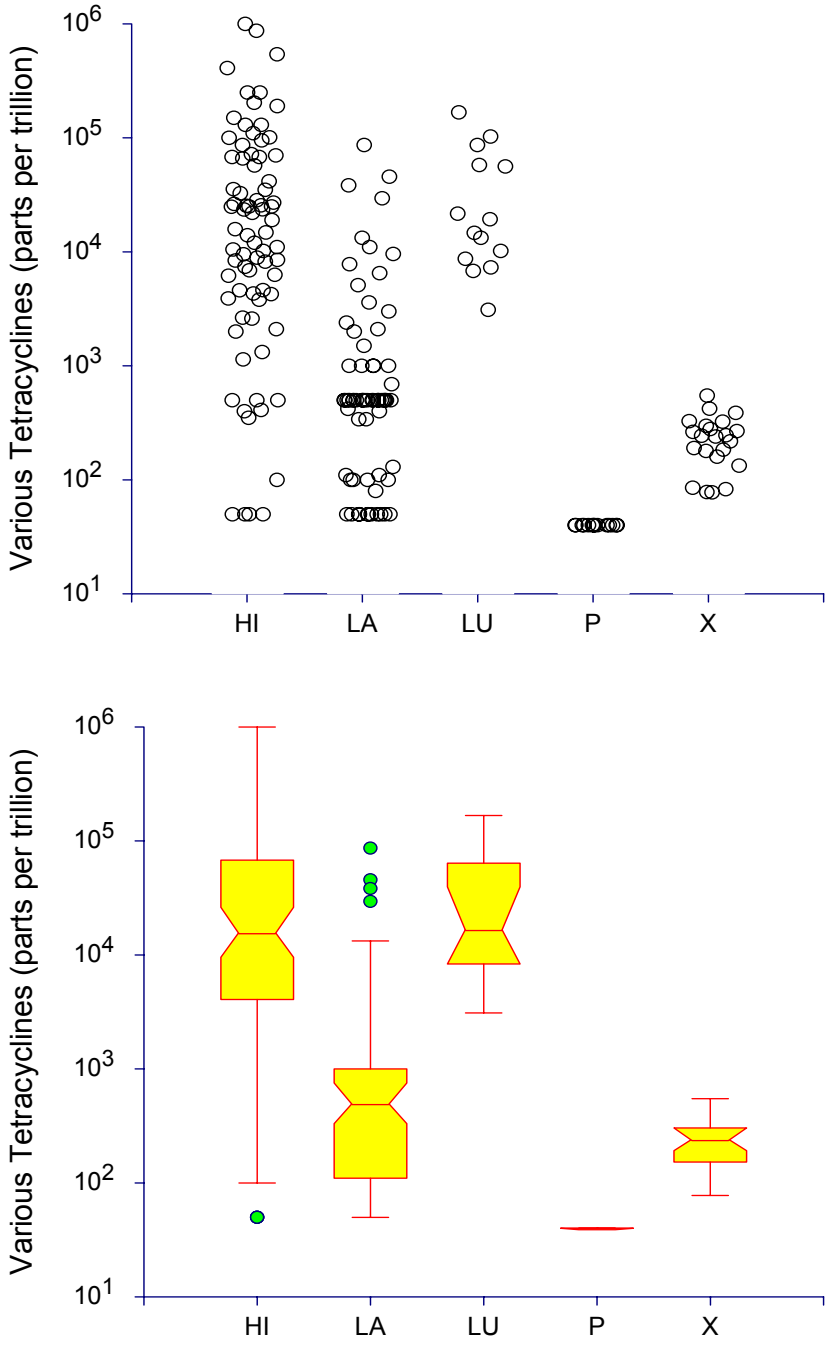

Figure 35. Contextual measurements of various individual tetracycline compounds (tetracycline, chlortetracycline, oxytetracycline, doxycycline, demeclocycline, meclocycline, and sum of tetracyclines).
Disturbance categories are (HI) heavy agriculture or heavy agriculture and urban, (LA) light agriculture or indeterminate location, (LU) urban, (P) pristine, and (X) experimental data from this survey.

Resistance Genes

Seven recent studies documented the relative abundance of resistance genes at targeted sites across a range of anthropogenic disturbance (Auerbach et al. 2007; Engemann et al. 2006; Mackie et al. 2006; Patterson et al. 2007; Peak et al. 2007; Pei et al. 2006; Pruden et al. 2006). By comparison, the ambient levels of *tetW* (Figure 36), *tetQ* (Figure 37), and *tetO* (Figure 38) observed in this study fall in the ranges previously observed in urban and light agricultural sites (see Appendix H – Contextual Values Of Tetracycline Resistance Genes (Raw Data)). In the case of tetW and tetO, observed ambient levels are also approximately five orders of magnitude lower than the highest values observed for sites categorized either as highly impacted or lightly impacted by urban disturbance. For tetQ, however, the difference between observed ambient levels and highly disturbed sites is much smaller (two orders of magnitude). Based on analysis of variance, the levels of all three resistance genes at highly impacted sites were significantly higher ($p<0.001$) than in all other groups, including the experimental observations of this study. Similarly, the ambient levels of all three genes observed in this study were not significantly different from the lightly impacted urban (LU), lightly impacted agriculture (LA), or pristine (P) sites, nor were the LU, LA, and P groups significantly different from each other.

Based on these results, there appears to be a small, but measurable background level of antibiotic resistance genes present not only in the observed study sites, but also in both lightly impacted and pristine sites previously targeted for study.

Even though this background level is significantly lower than highly impacted sites, it is evidence of a low-level reservoir of tetW, tetQ, and tetO in the environment. Further, this study measured resistance genes in the water column, and is therefore likely a conservative under-estimate of the numbers of genes present, since evidence for resistance gene migration into biofilms has been shown (Engemann et al. 2008). Whether the source of these genes is natural or introduced cannot be determined from this study, and further study with additional resistance genes as indicators may or may not yield similar results.

Nonetheless, the presence of a resistance reservoir holds several implications for antibiotic resistance. First, the genes are out there, and since they are both horizontally and vertically transferable, they are likely to persist once they are introduced. In addition, the co-selective nature of ribosomal protection suggests that the evolutionary cost-benefit of maintaining these genes may be higher than expected, since they provide protection from a broad spectrum of compounds, including metals. The mobility of the genetic elements themselves may also ameliorate the evolutionary cost, since these elements can be easily acquired by plasmid or transposon conjugation. The presence of the genes also suggests that addition of a strong antibiotic stressor (e.g., tetracycline or a metal) can rapidly shift the local microbial community towards resistant organisms. In other words, the ecological potential for widespread resistance is real.

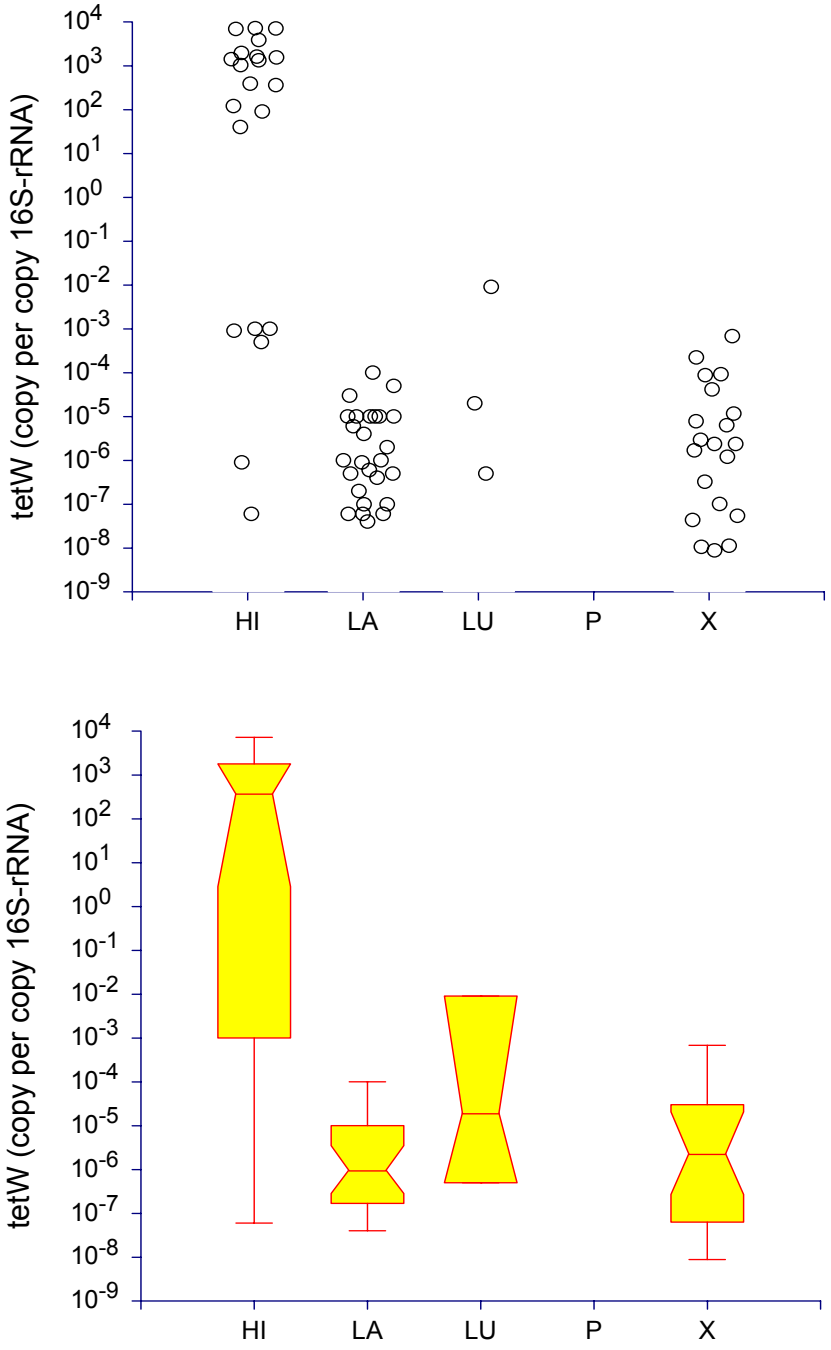

Figure 36. Contextual values tetW genes measured as relative proportions (copies per copy 16S-rRNA).
Disturbance categories are (HI) heavy agriculture or heavy agriculture and urban, (LA) light agriculture or indeterminate location, (LU) urban, (P) pristine, and (X) experimental data from this survey.

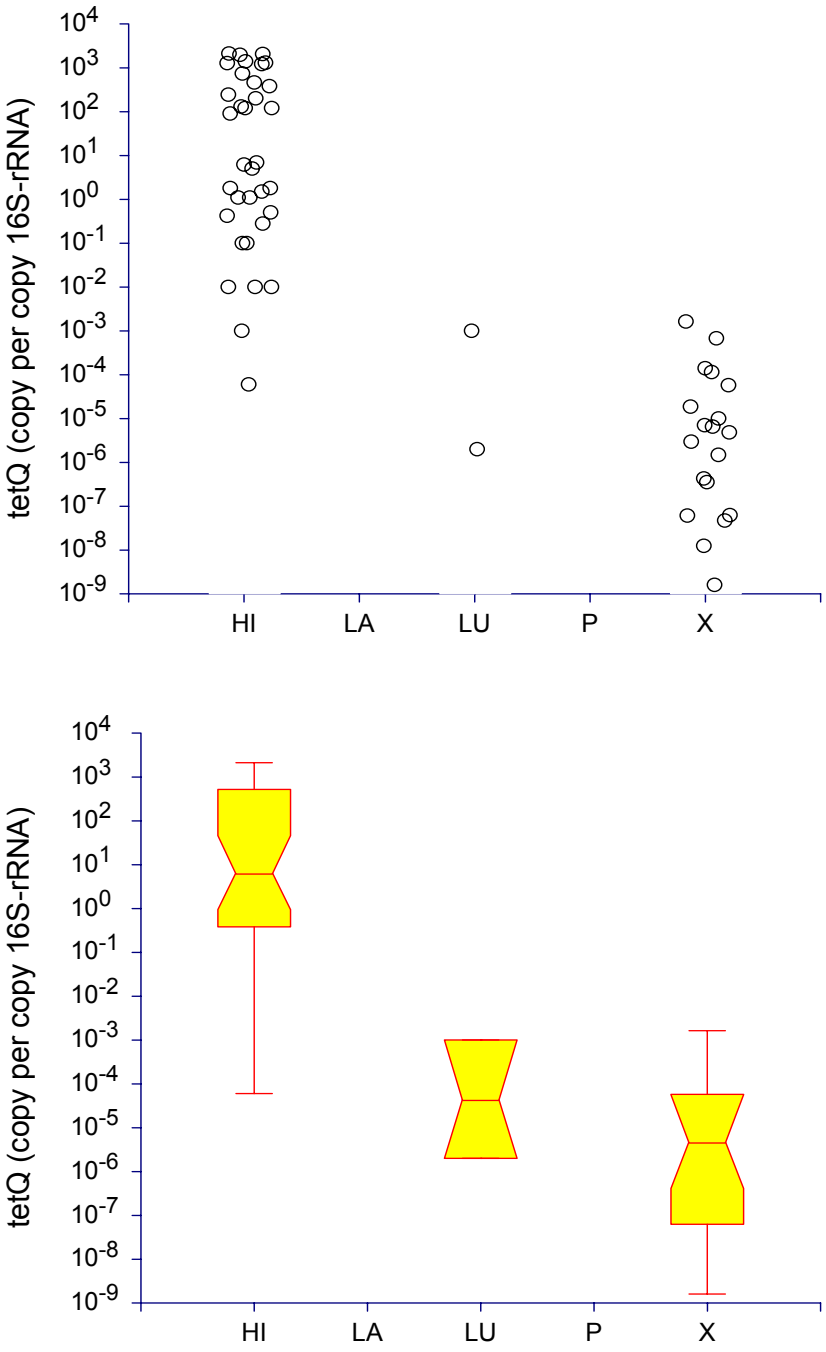

Figure 37. Contextual values tetQ genes measured as relative proportions (copies per copy 16S-rRNA).
Disturbance categories are (HI) heavy agriculture or heavy agriculture and urban, (LA) light agriculture or indeterminate location, (LU) urban, (P) pristine, and (X) experimental data from this survey.

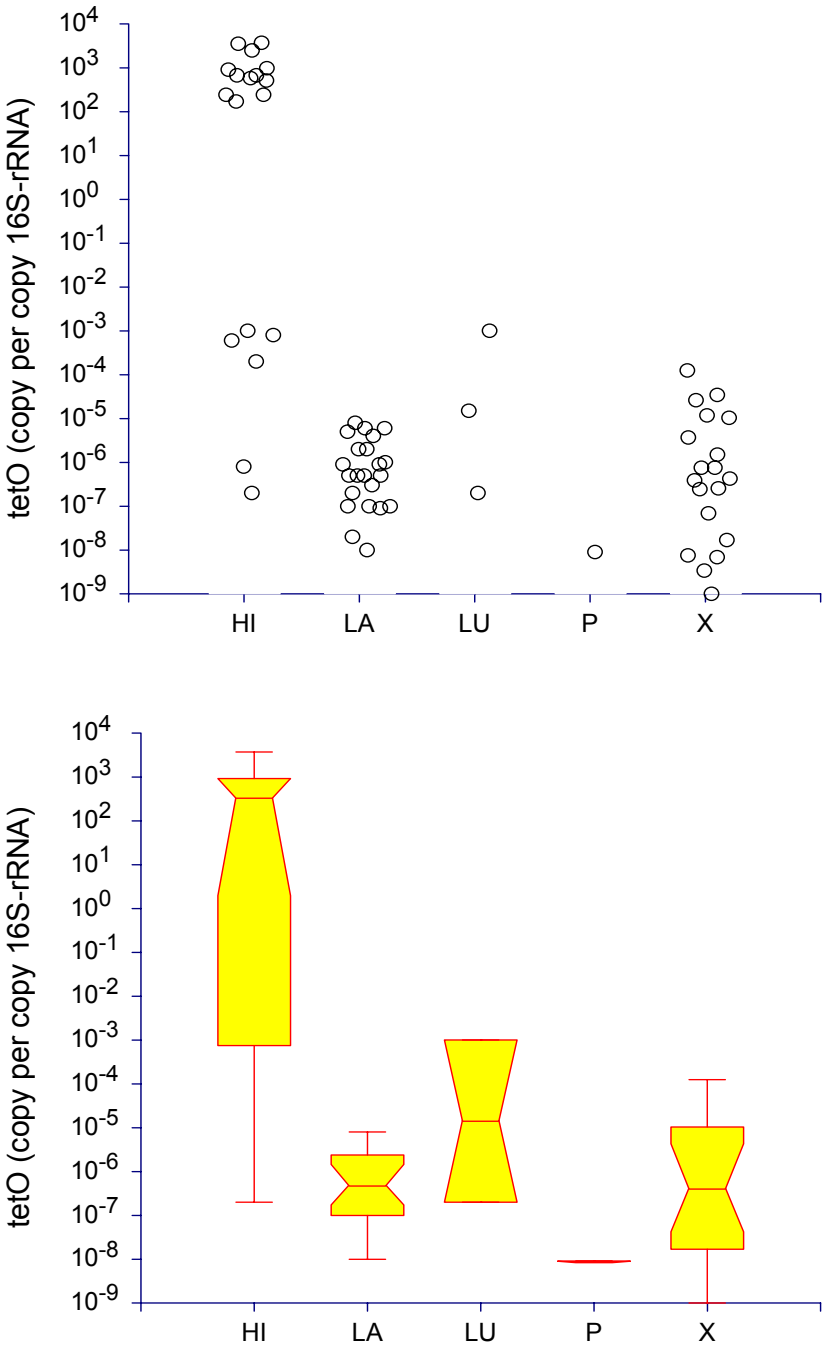

Figure 38. Contextual values tetO genes measured as relative proportions (copies per copy 16S-rRNA).
Disturbance categories are (HI) heavy agriculture or heavy agriculture and urban, (LA) light agriculture or indeterminate location, (LU) urban, (P) pristine, and (X) experimental data from this survey.

Evolutionary Cost of Resistance and Implications

Some types of antibiotic resistance genes provide protection from additional compounds. For example, ribosomal protection genes like tetW, tetQ, and tetO provide resistance to metals (Baker-Austin et al. 2006; Berg et al. 2005; Stepanauskas et al. 2006; Stepanauskas et al. 2005; Tuckfield and McArthur 2007; Wireman et al. 1997; Yurieva et al. 1997). These genes provide protection from multiple compounds, thereby becoming relatively more valuable than genes that code for resistance from a single compound or class of compounds. On an evolutionary timescale, added protection benefits reduce the evolutionary cost of maintaining the genes. In other words, over time, the benefit of maintaining the genes outweighs the energy and material expenditure for keeping them, and the time for reversion to sensitivity is inversely related to the cost of resistance (Spratt 1996). Moreover, the ability to transfer protection genes horizontally reduces the evolutionary cost, since the genes do not have to be maintained within the organism itself, so long as they are available via the local gene reservoir. In addition, the *ability* to transfer genes horizontally is generally favorable, because all sorts of beneficial traits can be inherited in this manner. If the genes were easily transferred and present, they would become rapidly more common under pressure from selection – a case which has been previously observed (Bruun et al. 2003; Salyers and Amabile-Cuevas 1997). Additionally, since selection pressure is episodic (e.g. through pulse introductions of antibiotics or metals in human and animal effluents), the evolutionary value of

maintaining the genes remains even in the absence of the selecting compound. Therefore, even low levels of antibiotics could be sufficient to maintain resistance. Evidence for episodic selection and the maintenance of a resistance gene reservoir has been shown in cases with animal feeding operations (Spratt 1996), hospitals (Salyers and Amabile-Cuevas 1997), and human gut (Salyers et al. 2004; Scott et al. 2000) and waste effluents (Lin et al. 2004). As noted by Baquero et al. (1998), an understanding of the dynamics and implications of antibiotic resistance will require understanding of the selective environments in which resistance develops.

Conclusions

According to the data gathered in this study, both tetracyclines and tetracycline resistance genes do occur at low, but measurable levels in perennial, wadeable streams of Kansas and Nebraska. In general, there appear to be no differences in tetracyclines, total gene counts, or resistance gene counts between Kansas and Nebraska, between reference and non-reference condition, or between Omernik Level III ecoregions. Too few sites were available to discern differences between USGS 4-digit hydrologic units. Despite the expected general similarity, some significant differences were observed, notably significant differences in both total abundance and relative proportion of tetW genes between Kansas and Nebraska, and Site 22 as a high outlier in both tetQ overall and tetO for the Central Irregular Plains ecoregion. Having Site 22 as an outlier is additionally surprising, considering it was one of the sites chosen by best professional judgment as reference or "least

impacted." Observed levels of tetracyclines, total gene counts, and the three resistance genes were in line with moderately impacted locations from previous studies, and in the case of tetracyclines, were statistically higher than locations previously characterized as pristine.

Environmental levels of tetracyclines did not correlate with total gene counts or with tetracycline resistance genes. However, the three tetracycline resistance genes (tetW, tetQ, and tetO) did significantly positively correlate with each other ($p<0.001$, $r^2 > 0.80$ for relative proportions of genes). This correlation is a novel finding and may imply either a similar mechanism for transfer or an environmental source reservoir (e.g., biofilms) for these three genes.

Statistically quantifiable estimates for the spatial extent of tetracyclines, total genes counts, and resistance gene counts (both total abundance and relative proportion) suggest that approximately 20% of the river kilometers in Kansas and Nebraska have measurable levels of these parameters *in situ*. This spatial extent, coupled with the contextual values estimates, suggests that an ambient reservoir of tetracyclines and tetracyclines resistance genes does exist in the environment, and that the extent of impairment (in terms of elevated tetracyclines or resistance gene counts) can be estimated both now and in the future using a linear spatial extent probability design. Additionally, the widespread presence of low levels of tetracycline and tetracycline resistance genes suggest that tetracycline resistance could be evolutionarily favored and persistent through time in these systems.

Future work could benefit from the data gathered in this study in three specific ways. First, additional analyses of watershed and other concurrently collected data may reveal correlations with the parameters considered in this study and help to understand the selective environment of Kansas and Nebraska streams. Second, the probability design framework could be used to calibrate predictive risk models developed by analysis of concurrently collected data, especially human population density, cropland, and CAFO size and location. Finally, the baseline data provided by this study could be used as the starting point in a short or long term monitoring study to determine the change (if there is any) in antibiotic concentrations and resistance gene prevalence in streams of Kansas and Nebraska.

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Appendix A – Selected Watershed and Site Characteristics

Table A-1. Landscape characteristics by site. Watershed area and percent dominant land cover were calculated from watersheds delineated as part of this project using existing geospatial data from the Kansas Applied Remote Sensing Program of the Kansas Biological Survey. Road density and population density were calculated as part of the National Wadeable Stream Assessment (USEPA 2006). Ref: best professional judgment "reference" site; Non: probability selected "non-reference" site. NGP: Northern Great Plains; WHP: Western High Plains; NSH: Northern Sand Hills; NGL: Northern Glaciated Plains; WCB: Western Corn Belt Plains; CGP: Central Great Plains; ST: Southwest Tablelands; COT: Central Oklahoma Tablelands; CIP: Central Irregular Plains; OH: Ozark Highlands.

							% Dominant Land Cover				
Site Number	State	Reference	Ecoregion	Hydrologic Unit Code	Sample Collection Date	Watershed Area (sq. km)	Cropland	Pasture	Grassland	Road Density (km per km2)	Population Density (count per km2)
1	NE	Non	WCB	1020	07/14/04	236	81.1	12.3	5.1	1.49	7
2	KS	Non	CIP	1107	09/14/04	36.1	25.4	64.0	4.7	1.52	12
3	KS	Non	CIP	1029	09/16/04	27.3	43.7	27.0	18.8	1.41	2
4	NE	Non	CGP	1020	09/17/04	180	78.9	7.1	12.3	1.26	2
5	KS	Non	SWT	1106	09/28/04	169	33.7	4.3	56.6	1.04	0
6	KS	Non	CGP	1103	09/30/04	30.7	46.6	22.5	29.1	1.43	3
7	NE	Non	WCB	1017	06/22/04	166	62.7	28.1	6.2	1.47	7
8	NE	Non	WHP	1014	06/20/04	114	0.2	0.2	99.3	0.82	0
9	NE	Non	WCB	1022	07/15/04	52.0	88.9	8.2	2.3	1.32	1
10	KS	Non	CGP	1106	09/29/04	140	49.9	8.3	38.9	1.26	1
11	KS	Non	CGP	1025	09/20/04	167	48.7	5.8	38.0	1.27	1
12	KS	Non	CIP	1029	08/25/04	102	6.4	21.4	63.9	0.76	1
13	NE	Non	WCB	1024	07/13/04	63.3	57.6	17.1	17.3	1.37	2
14	NE	Non	WCB	1023	06/23/04	7.90	56.4	37.4	0.0	1.43	8
15	NE	Non	NGP	1015	07/19/04	121	8.4	1.8	87.1	1.5	0
16	KS	Non	CGP	1026	09/21/04	21.4	19.8	6.5	70.5	0.73	1
17	KS	Non	FH	1027	09/08/04	12.8	20.1	22.0	51.3	1.42	2
18	KS	Ref	FH	1107	09/10/04	294	7.4	11.9	75.5	0.94	1
19	KS	Ref	FH	1027	09/24/04	89.8	4.9	19.3	64.0	0.6	1
20	KS	Ref	SWT	1106	09/22/04	23.0	17.1	5.8	73.7	1.03	0
21	NE	Ref	CGP	1025	10/24/04	123	30.2	12.8	52.4	1.36	1
22	NE	Ref	CGP	1025	10/25/04	38.7	16.4	2.4	78.0	0.7	1

Table A-2. Observed chemical parameters by site. Parameters were sampled, processed, and measured according to field (USEPA 2004b), laboratory (USEPA 2004a; USEPA 2004c), and quality assurance methods (USEPA 2004d) of the National Wadeable Stream Assessment (USEPA 2006). In cases of multiple visits, values were averaged.

Site Number	Percent Channel Cover	Stream Temperature (deg C)	Flow (cfs)	pH	Conductivity (uS/cm)	Turbidity (NTU)	Total Suspended Solids (mg/L)
1	17.9	18.7	7.35	7.86	521.5	42.75	69
2	91.3	18.25	0.00	7.435	299	13.645	36.5
3	84.2	19	0.00	7.07	177	391	260.1
4	0.1	19.4	3.30	8.14	1190	6.5	14
5	56.4	18	7.37	8.19	526	6.03	5.4
6	99.9	15	0.00	7.45	1934	4.86	10.2
7	28.1	16.3	9.26	8.19	1051	8.5	33.8
8	12.2	14	24.58	8.25	304	0.476	0.3
9	23.1	22.5	0.22	7.1	256	1516	109.6
10	69.8	16	10.79	8.09	466	22.4	44.8
11	68.8	23	0.00	7.89	2642	63.5	98.8
12	48.8	24	8.16	7.95	506	2.79	21.9
13	73.2	26.1	0.17	7.88	515	12.4	11.3
14	36.0	16.3	1.30	8.17	702	28.4	56.2
15	84.5	12.95	0.36	7.675	346	0.268	0.95
16	95.6	21	0.00	7.77	1047	62.2	160
17	96.1	24	0.00	7.81	473	182	226.4
18	63.0	20	13.26	7.98	515	10.2	9.8
19	65.8	22	0.93	8	574	0.499	2.8
20	34.2	19	9.00	8.23	509	1.92	18
21	51.3	9.5	2.09	7.85	655	1.66	6
22	17.6	16.7	3.50	8.21	509	1.19	21.4

Table A-2 (continued). Observed chemical parameters by site. In cases of multiple visits, values were averaged.

Site Number	Dissolved Organic Carbon (mg/L)	Dissolved Inorganic Carbon (mg/L)	Total Phosphorus (µg/L)	Total Nitrogen (mg/L)	Dissolved Selenium (µg/L)	Dissolved Zinc (µg/L)	Calculated Alkalinity (ueq/L)	Sum of Cations (ueq/L)
1	3.335	46.975	410.5	2678	1.75	0	3810	5560
2	7.415	34.98	137.5	709	0.25	17.9	2690	2990
3	8.35	17.61	608	1969	0	51.7	1230	1650
4	7.14	43.74	255	2233	0	34.6	3610	13370
5	1.15	43.25	24	1956	0.4	0	3580	5270
6	4.04	71.05	165	403	0.9	0	5490	23450
7	3.16	62.3	313	43650	0	10	5150	11850
8	1.58	36.38	16	1114	0	16.1	3020	3330
9	12.64	26.76	1424	5781	0	0	1890	2680
10	1.74	48.29	34	4800	0	0	3970	4770
11	4.71	68.35	316	1265	0	45.3	5550	25200
12	2.63	44	41	454	0	14.8	3590	5360
13	3.74	55.3	139	716	0	0	4490	5660
14	2.84	77.21	130	3881	7.5	0	6380	8480
15	1.645	41.91	14	706	0.45	0	3340	3690
16	5.63	68.17	172	784	0.3	22.7	5480	10760
17	4.91	52.5	420	1889	0	2.2	4240	5460
18	1.98	58.11	45	604	0.2	0	4750	5510
19	1.55	61.64	17	203	0	0	5040	6330
20	0.9	49.41	16	2534	0.8	0	4090	5160
21	3.6	56.77	243	1218	0	0	4600	6990
22	1.73	52.39	127	3384	0.7	0	4340	5610

Appendix B – Observed Values By USGS 4-Digit Hydrologic Unit Code

Table B-1. Summary of tetracyclines, genes, and resistance genes by hydrologic unit.

		Count	Mean	Standard Deviation	Standard Error	Minimum	Maximum	Median
Genes (copies per mL)								
tetW	*1014*	2	5.08	2.71	1.91	3.16	6.99	5.08
	1015	3	24.6	17.4	10.1	10.4	44.0	19.4
	1017	3	193	82.2	47.4	130	285	162
	1020	6	4.97	4.05	1.65	1.05	8.95	4.91
	1022	1	154	0	0	154	154	154
	1023	3	0.950	0.201	0.116	0.719	1.08	1.05
	1024	2	1.13	0.970	0.686	0.444	1.82	1.13
	1025	9	73.4	105	34.9	1.63	278	15.4
	1026	3	1.76	1.52	0.878	0.198	3.24	1.86
	1027	1	0.106	0	0	0.106	0.106	0.106
	1029	5	3.09	2.50	1.12	0.0458	5.68	3.11
	1103	2	0.394	0.367	0.259	0.134	0.653	0.394
	1106	7	8.88	7.92	2.99	0.966	23.1	9.16
	1107	4	0.937	0.991	0.495	0.171	2.38	0.597
tetQ	*1014*	2	13.8	10.7	7.59	6.18	21.4	13.8
	1015	2	6.12	1.50	1.06	5.06	7.18	6.12
	1017	3	307	243	140	74.9	559	288
	1020	6	10.5	10.6	4.32	1.23	26.3	5.97
	1022	0	0	0	0	0	0	0
	1023	3	4.05	0.926	0.534	2.99	4.68	4.48
	1024	2	1.89	2.57	1.82	0.0668	3.71	1.89
	1025	9	218	311	104	1.87	839	29.9
	1026	3	2.59	4.03	2.33	0.155	7.24	0.385
	1027	0	0	0	0	0	0	0
	1029	5	5.73	4.19	1.87	1.47	11.4	7.06
	1103	1	1.62	0	0	1.62	1.62	1.62
	1106	7	39.9	48.5	18.3	5.55	140	23.9
	1107	5	0.704	0.633	0.283	0.138	1.79	0.546

Table B-1 (continued). Summary of tetracyclines, genes, and resistance genes by hydrologic unit.

		Count	Mean	Standard Deviation	Standard Error	Minimum	Maximum	Median
tetO	*1014*	2	0.496	0.558	0.394	0.101	0.890	0.496
	1015	3	1.42	0.750	0.433	0.556	1.88	1.82
	1017	3	18.6	7.74	4.47	11.0	26.5	18.4
	1020	5	0.871	0.595	0.266	0.251	1.62	0.674
	1022	1	20.6	0	0	20.6	20.6	20.6
	1023	3	0.160	0.0134	0.00773	0.145	0.171	0.163
	1024	2	0.113	0.100	0.0711	0.0420	0.184	0.113
	1025	9	6.34	7.97	2.66	0.126	24.3	3.43
	1026	3	0.674	0.491	0.284	0.140	1.11	0.776
	1027	0	0	0	0	0	0	0
	1029	5	0.560	0.256	0.114	0.185	0.809	0.642
	1103	1	0.731	0	0	0.731	0.731	0.731
	1106	7	1.31	0.910	0.344	0.175	2.80	1.02
	1107	2	0.0535	0.0175	0.0124	0.0411	0.0660	0.0535
Genes normalized to 16s-rRNA								
tetW	*1014*	2	6.31E-06	7.37E-06	5.21E-06	1.10E-06	1.15E-05	6.31E-06
	1015	3	2.93E-06	2.98E-06	1.72E-06	9.62E-07	6.37E-06	1.47E-06
	1017	3	7.82E-06	2.96E-06	1.71E-06	4.62E-06	1.04E-05	8.41E-06
	1020	6	3.39E-04	4.30E-04	1.75E-04	4.52E-08	1.03E-03	1.70E-04
	1022	1	8.77E-05	0	0	8.77E-05	8.77E-05	8.77E-05
	1023	3	1.01E-07	4.35E-08	2.51E-08	6.28E-08	1.49E-07	9.26E-08
	1024	1	1.06E-08	0	0	1.06E-08	1.06E-08	1.06E-08
	1025	6	1.44E-05	2.62E-05	1.07E-05	8.77E-08	6.63E-05	1.58E-06
	1026	3	8.85E-09	0	0	8.59E-10	1.56E-08	1.01E-08
	1027	0	0	0	0	0	0	0
	1029	4	6.89E-05	1.37E-04	6.87E-05	4.36E-08	2.75E-04	3.22E-07
	1103	2	1.16E-05	1.64E-05	1.16E-05	5.20E-08	2.32E-05	1.16E-05
	1106	6	1.12E-04	1.84E-04	7.53E-05	1.20E-06	4.73E-04	3.19E-05
	1107	3	3.99E-08	3.82E-08	2.20E-08	1.13E-08	8.32E-08	2.51E-08

Table B-1 (continued). Summary of tetracyclines, genes, and resistance genes by hydrologic unit.

		Count	Mean	Standard Deviation	Standard Error	Minimum	Maximum	Median
tetQ	*1014*	2	1.87E-05	2.34E-05	1.65E-05	2.16E-06	3.52E-05	1.87E-05
	1015	2	3.55E-07	1.63E-07	1.15E-07	2.40E-07	4.70E-07	3.55E-07
	1017	3	1.00E-05	4.52E-06	2.61E-06	6.04E-06	1.49E-05	9.05E-06
	1020	6	8.19E-04	1.15E-03	4.70E-04	6.08E-08	2.52E-03	1.74E-04
	1022	0	0	0	0	0	0	0
	1023	3	4.30E-07	1.77E-07	1.02E-07	2.64E-07	6.16E-07	4.09E-07
	1024	1	1.60E-09	0	0	1.60E-09	1.60E-09	1.60E-09
	1025	6	4.80E-05	8.10E-05	3.31E-05	1.01E-07	2.00E-04	3.35E-06
	1026	3	1.25E-08	1.93E-08	1.12E-08	6.75E-10	3.48E-08	2.10E-09
	1027	0	0	0	0	0	0	0
	1029	4	8.61E-05	1.71E-04	8.57E-05	6.12E-08	3.43E-04	6.20E-07
	1103	1	5.77E-05	0	0	5.77E-05	5.77E-05	5.77E-05
	1106	6	3.41E-04	4.77E-04	1.95E-04	4.05E-06	1.23E-03	1.94E-04
	1107	4	5.49E-08	4.89E-08	2.44E-08	9.11E-09	1.16E-07	4.70E-08
tetO	*1014*	2	7.51E-07	1.01E-06	7.16E-07	3.54E-08	1.47E-06	7.51E-07
	1015	3	2.44E-07	3.24E-07	1.87E-07	5.16E-08	6.18E-07	6.09E-08
	1017	3	7.56E-07	2.86E-07	1.65E-07	4.28E-07	9.55E-07	8.85E-07
	1020	5	5.06E-05	8.49E-05	3.80E-05	8.30E-09	1.98E-04	4.46E-06
	1022	1	1.17E-05	0	0	1.17E-05	1.17E-05	1.17E-05
	1023	3	1.69E-08	0	0	1.28E-08	2.35E-08	1.42E-08
	1024	1	1.01E-09	0	0	1.01E-09	1.01E-09	1.01E-09
	1025	6	1.33E-06	2.27E-06	9.25E-07	6.81E-09	5.79E-06	2.82E-07
	1026	3	3.39E-09	0	0	6.07E-10	5.32E-09	4.24E-09
	1027	0	0	0	0	0	0	0
	1029	4	7.80E-06	1.55E-05	7.76E-06	6.84E-09	3.11E-05	5.46E-08
	1103	1	2.60E-05	0	0	2.60E-05	2.60E-05	2.60E-05
	1106	6	1.74E-05	2.30E-05	9.40E-06	2.25E-07	5.24E-05	5.81E-06
	1107	1	7.56E-09	0	0	7.56E-09	7.56E-09	7.56E-09

Table B-1 (continued). Summary of tetracyclines, genes, and resistance genes by hydrologic unit.

		Count	Mean	Standard Deviation	Standard Error	Minimum	Maximum	Median
Tetracyclines (parts per trillion)								
TCs	*1014*	1	160	0	0	160	160	160
	1015	1	548	0	0	548	548	548
	1017	1	190	0	0	190	190	190
	1020	2	260	180	127	133	387	260
	1022	1	298	0	0	298	298	298
	1023	1	264	0	0	264	264	264
	1024	1	244	0	0	244	244	244
	1025	3	144	108	62.1	78.1	268	85.4
	1026	1	324	0	0	324	324	324
	1027	2	129	71.8	50.8	77.8	179	129
	1029	2	284	61.4	43.5	240	327	284
	1103	1	218	0	0	218	218	218
	1106	3	237	48.4	27.9	184	280	246
	1107	2	252	239	169	82.9	421	252
Genes (copies per mL)								
16s-rRNA	*1014*	3	4.27E+06	4.54E+06	2.62E+06	6.07E+05	9.35E+06	2.86E+06
	1015	3	1.46E+07	1.38E+07	7.99E+06	3.05E+06	2.99E+07	1.08E+07
	1017	3	3.12E+07	2.67E+07	1.54E+07	1.24E+07	6.18E+07	1.93E+07
	1020	6	8.46E+06	1.34E+07	5.47E+06	8.20E+03	3.03E+07	8.89E+04
	1022	3	2.34E+06	7.36E+05	4.25E+05	1.76E+06	3.17E+06	2.10E+06
	1023	3	1.00E+07	2.38E+06	1.38E+06	7.28E+06	1.15E+07	1.13E+07
	1024	1	4.17E+07	0	0	4.17E+07	4.17E+07	4.17E+07
	1025	6	1.60E+07	1.17E+07	4.77E+06	4.19E+06	3.63E+07	1.43E+07
	1026	3	2.07E+08	2.35E+07	1.35E+07	1.83E+08	2.30E+08	2.08E+08
	1027	3	6.84E+05	5.72E+05	3.30E+05	2.51E+04	1.06E+06	9.70E+05
	1029	4	1.42E+07	1.14E+07	5.71E+06	2.07E+04	2.71E+07	1.49E+07
	1103	3	8.80E+05	1.47E+06	8.51E+05	2.81E+04	2.58E+06	3.14E+04
	1106	6	2.59E+06	3.57E+06	1.46E+06	1.56E+04	7.18E+06	5.88E+05
	1107	5	9.44E+06	7.55E+06	3.38E+06	2.40E+04	1.86E+07	8.73E+06

Table B-1 (continued). Summary of tetracyclines, genes, and resistance genes by hydrologic unit.

		Count	Mean	Standard Deviation	Standard Error	Minimum	Maximum	Median
Genes (copies per mL)								
tetR	*1014*	2	19.3	14.0	9.90	9.44	29.2	19.3
	1015	3	30.1	20.0	11.6	16.0	53.0	21.3
	1017	3	519	331	191	216	871	469
	1020	6	16.1	14.2	5.82	3.08	35.0	12.0
	1022	1	175	0	0	175	175	175
	1023	3	5.16	0.850	0.491	4.19	5.74	5.57
	1024	2	3.13	3.64	2.58	0.552	5.71	3.13
	1025	9	298	419	140	3.63	1140	57.8
	1026	3	5.03	5.81	3.36	0.492	11.6	3.02
	1027	1	0.106	0	0	0.106	0.106	0.106
	1029	5	9.38	6.36	2.84	1.94	15.3	13.2
	1103	2	1.57	2.03	1.44	0.134	3.01	1.57
	1106	7	50.1	57.0	21.5	7.06	165	34.0
	1107	5	1.47	1.59	0.709	0.309	4.21	0.891
Genes normalized to 16s-rRNA								
tetR	*1014*	2	2.57E-05	3.17E-05	2.24E-05	3.30E-06	4.82E-05	2.57E-05
	1015	3	3.41E-06	3.10E-06	1.79E-06	1.48E-06	6.98E-06	1.77E-06
	1017	3	1.86E-05	5.20E-06	3.00E-06	1.41E-05	2.43E-05	1.74E-05
	1020	6	1.20E-03	1.63E-03	6.64E-04	1.38E-07	3.74E-03	3.72E-04
	1022	1	9.94E-05	0	0	9.94E-05	9.94E-05	9.94E-05
	1023	3	5.48E-07	2.16E-07	1.25E-07	3.69E-07	7.88E-07	4.86E-07
	1024	1	1.33E-08	0	0	1.33E-08	1.33E-08	1.33E-08
	1025	6	6.37E-05	1.09E-04	4.46E-05	1.95E-07	2.73E-04	5.21E-06
	1026	3	2.48E-08	2.77E-08	1.60E-08	2.14E-09	5.57E-08	1.65E-08
	1027	0	0	0	0	0	0	0
	1029	4	1.63E-04	3.24E-04	1.62E-04	1.12E-07	6.49E-04	9.97E-07
	1103	2	5.35E-05	7.56E-05	5.34E-05	5.20E-08	1.07E-04	5.35E-05
	1106	6	4.70E-04	6.80E-04	2.78E-04	5.76E-06	1.76E-03	2.32E-04
	1107	4	8.67E-08	6.45E-08	3.22E-08	2.04E-08	1.62E-07	8.21E-08

Appendix C – Total Gene Counts Observed Values Figures

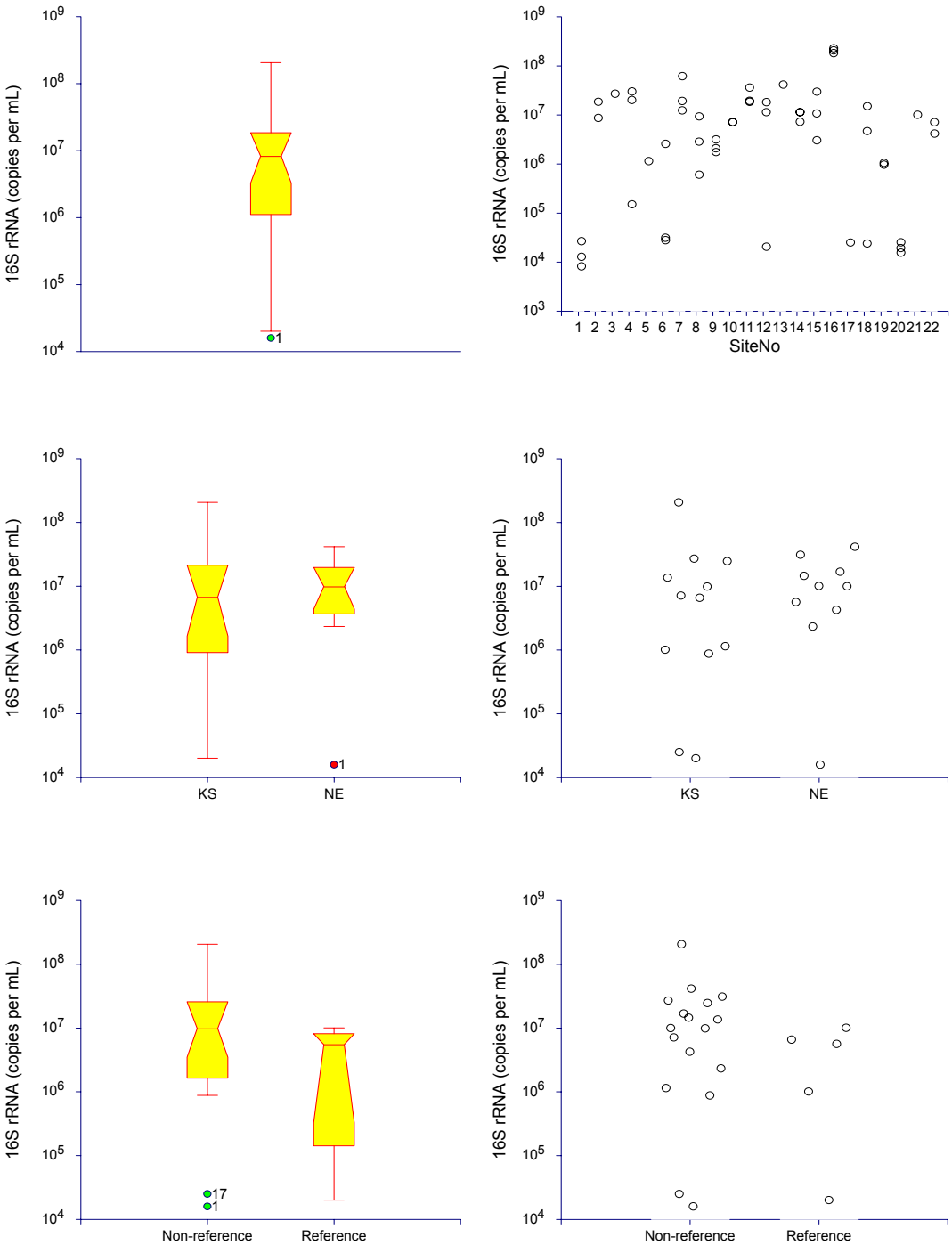

Figure C-1. Summary plots for total gene (16S-rRNA) counts: box plots (a) for all measurements, (b) by state, and (c) by reference category; and dot plots (d) for all measurements, (e) by state, and (f) by reference category.

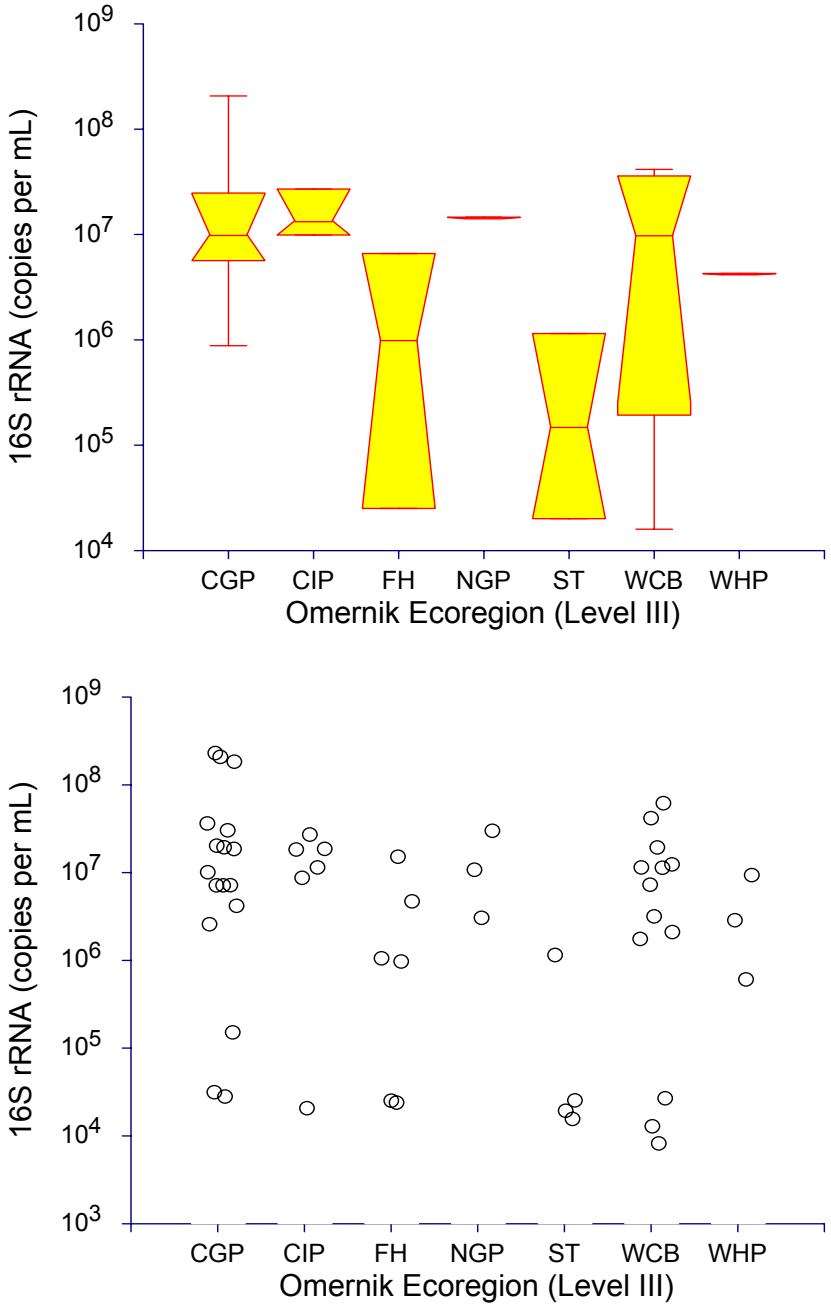

Figure C-2. Box plot and dot plot of total gene (16SrRNA) counts by Omernik Level III Ecoregion. NGP: Northern Great Plains; WHP: Western High Plains; NSH: Northern Sand Hills; NGL: Northern Glaciated Plains; WCB: Western Corn Belt Plains; CGP: Central Great Plains; ST: Southwest Tablelands; COT: Central Oklahoma Tablelands; CIP: Central Irregular Plains; OH: Ozark Highlands.

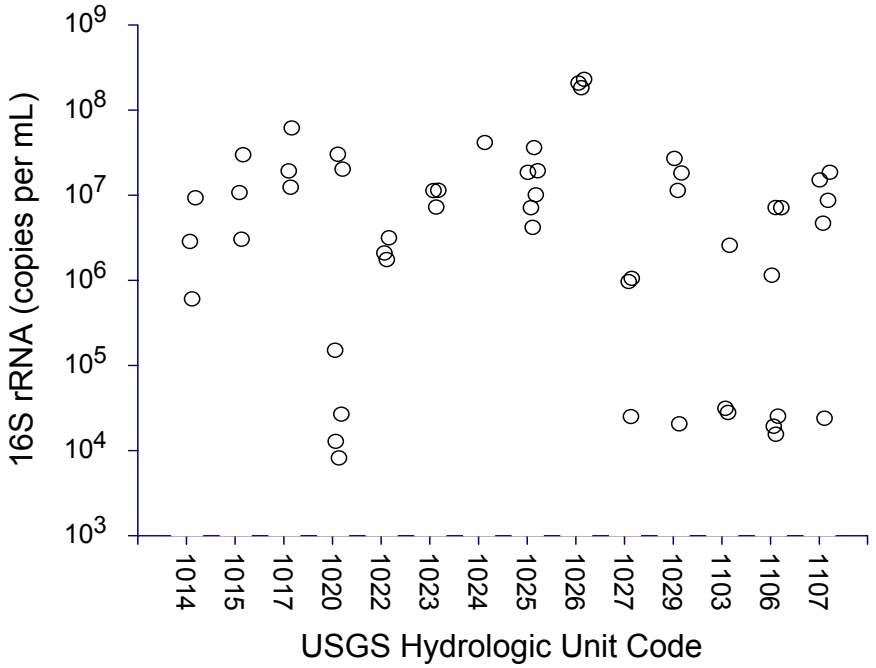

Figure C-3. Dot plot of total gene (16SrRNA) counts by 4 digit USGS Hydrologic Unit Code.

Appendix D – tetQ Observed Values Figures

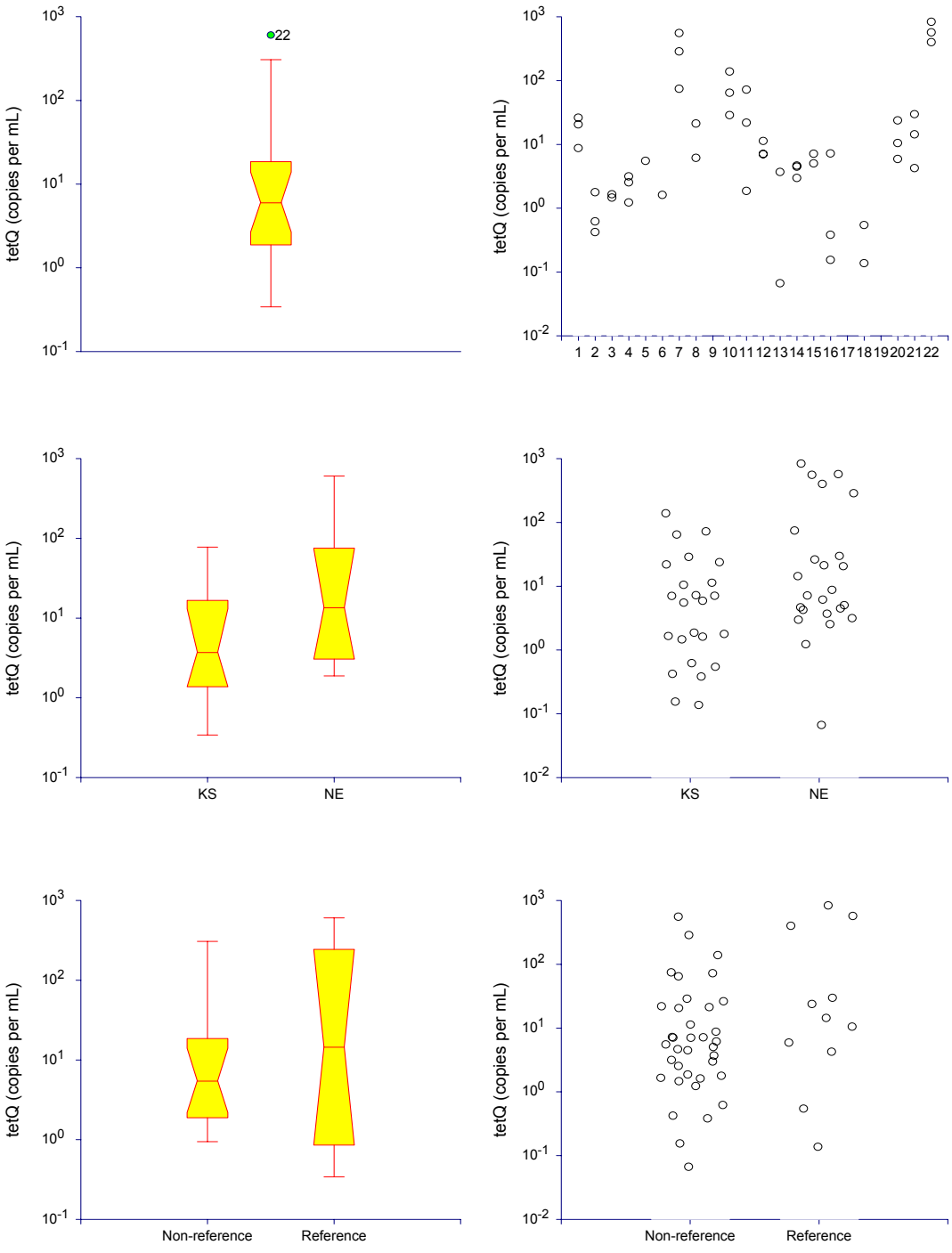

Figure D-1. Summary plots for tetQ gene counts: box plots (a) for all measurements, (b) by state, and (c) by reference category; and dot plots (d) for all measurements, (e) by state, and (f) by reference category.

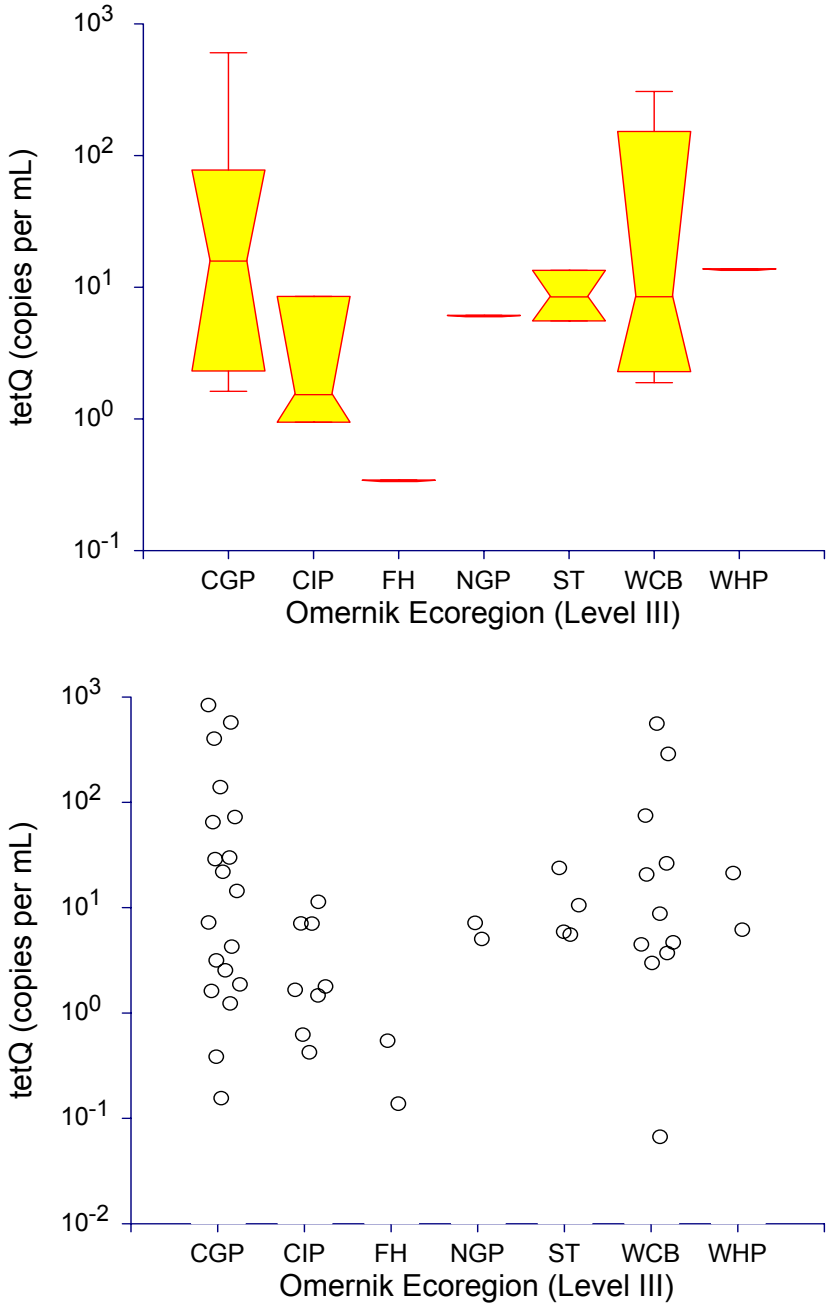

Figure D-2. Box plot and dot plot of tetQ gene counts by Omernik Level III Ecoregion. NGP: Northern Great Plains; WHP: Western High Plains; NSH: Northern Sand Hills; NGL: Northern Glaciated Plains; WCB: Western Corn Belt Plains; CGP: Central Great Plains; ST: Southwest Tablelands; COT: Central Oklahoma Tablelands; CIP: Central Irregular Plains; OH: Ozark Highlands.

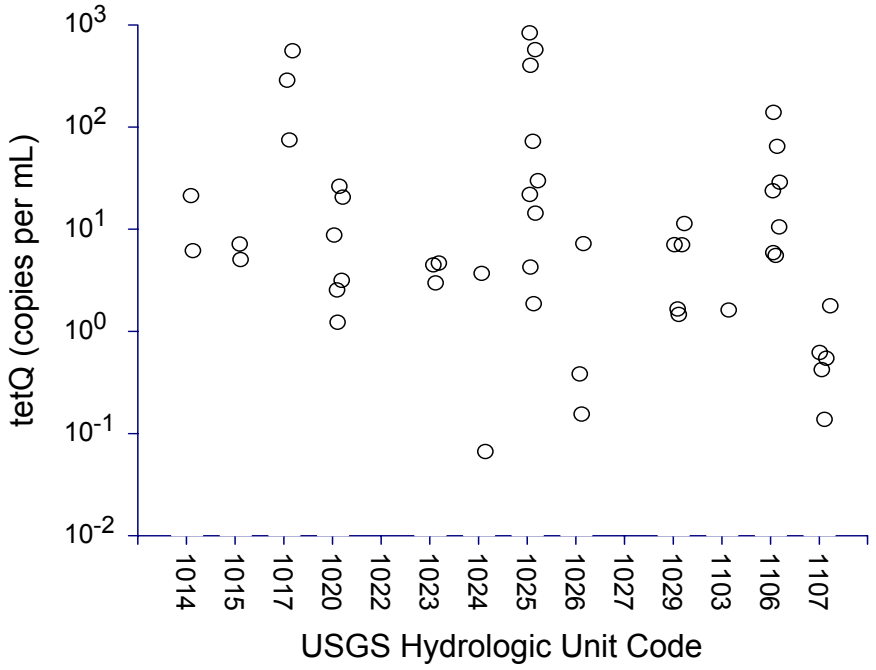

Figure D-3. Dot plot of tetQ gene counts by 4 digit USGS Hydrologic Unit Code.

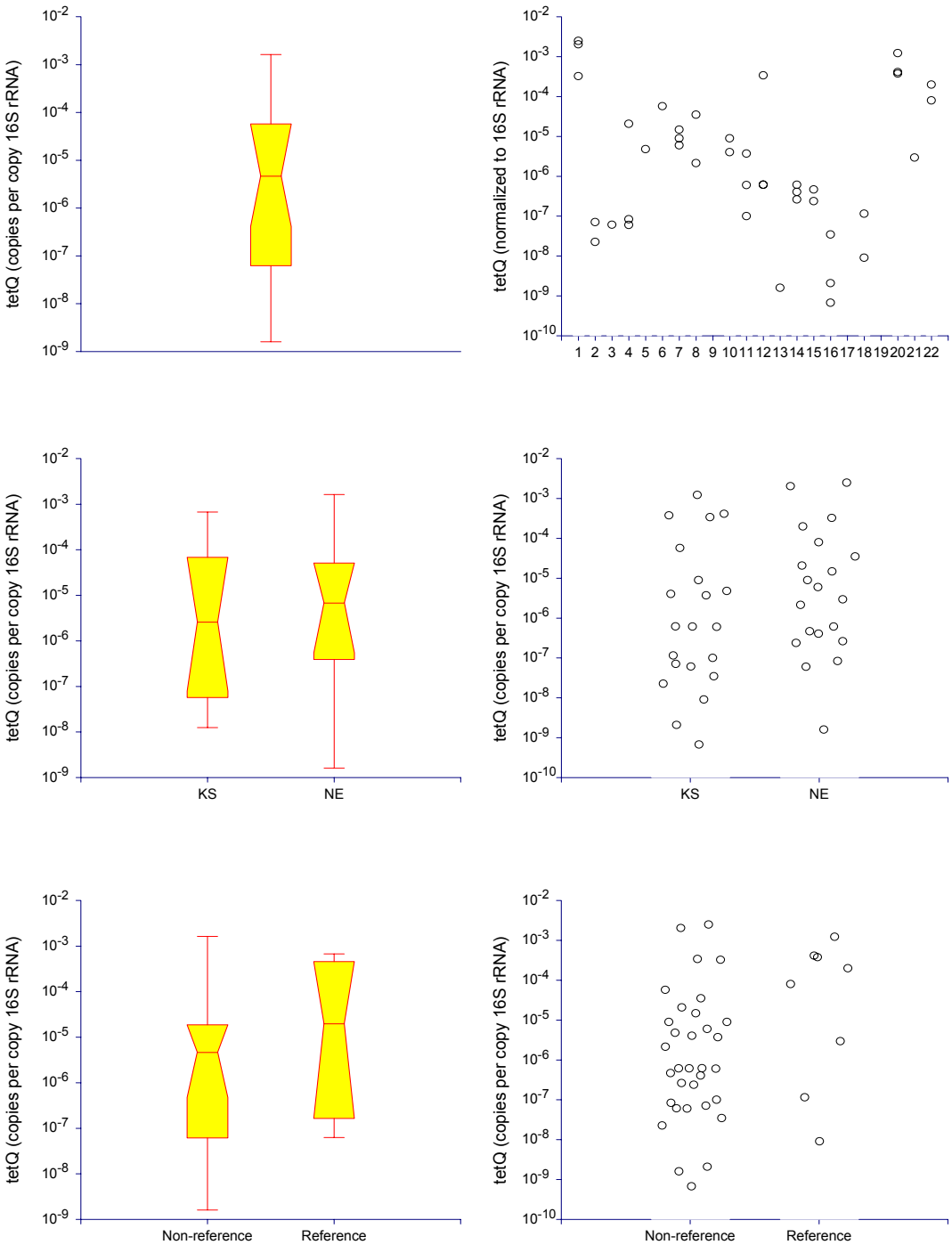

Figure D-4. Summary plots for tetQ gene counts relative to 16SrRNA: box plots (a) for all measurements, (b) by state, and (c) by reference category; and dot plots (d) for all measurements, (e) by state, and (f) by reference category.

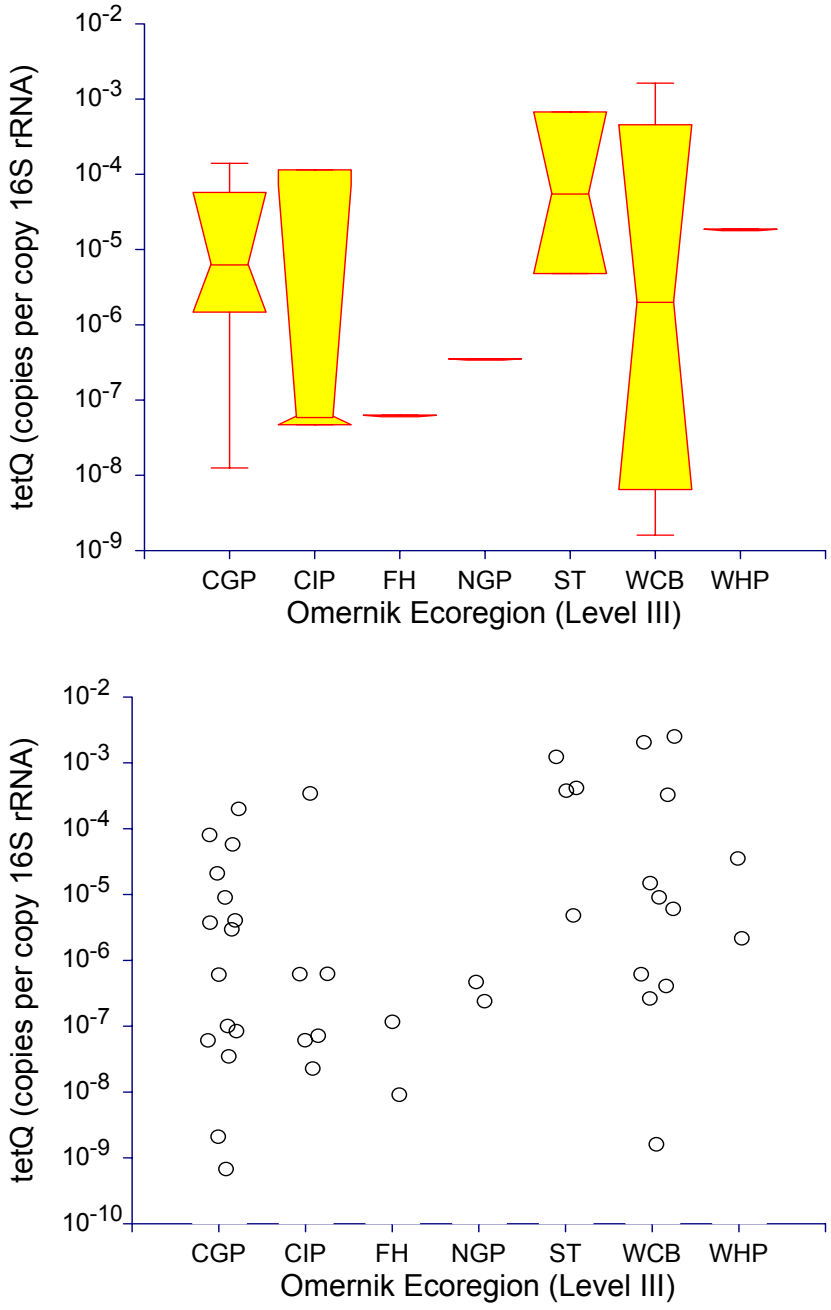

Figure D-5. Box plot and dot plot of tetQ total gene counts relative to 16SrRNA by Omernik Level III Ecoregion. NGP: Northern Great Plains; WHP: Western High Plains; NSH: Northern Sand Hills; NGL: Northern Glaciated Plains; WCB: Western Corn Belt Plains; CGP: Central Great Plains; ST: Southwest Tablelands; COT: Central Oklahoma Tablelands; CIP: Central Irregular Plains; OH: Ozark Highlands.

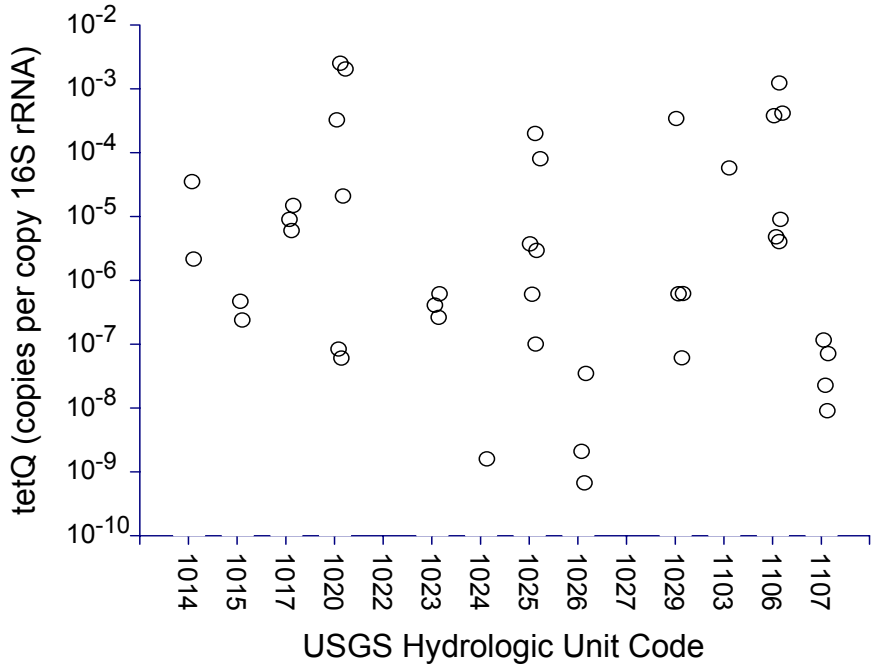

Figure D-6. Dot plot of tetQ gene counts relative to 16SrRNA by 4 digit USGS Hydrologic Unit Code.

Appendix E – tetO Observed Values Figures

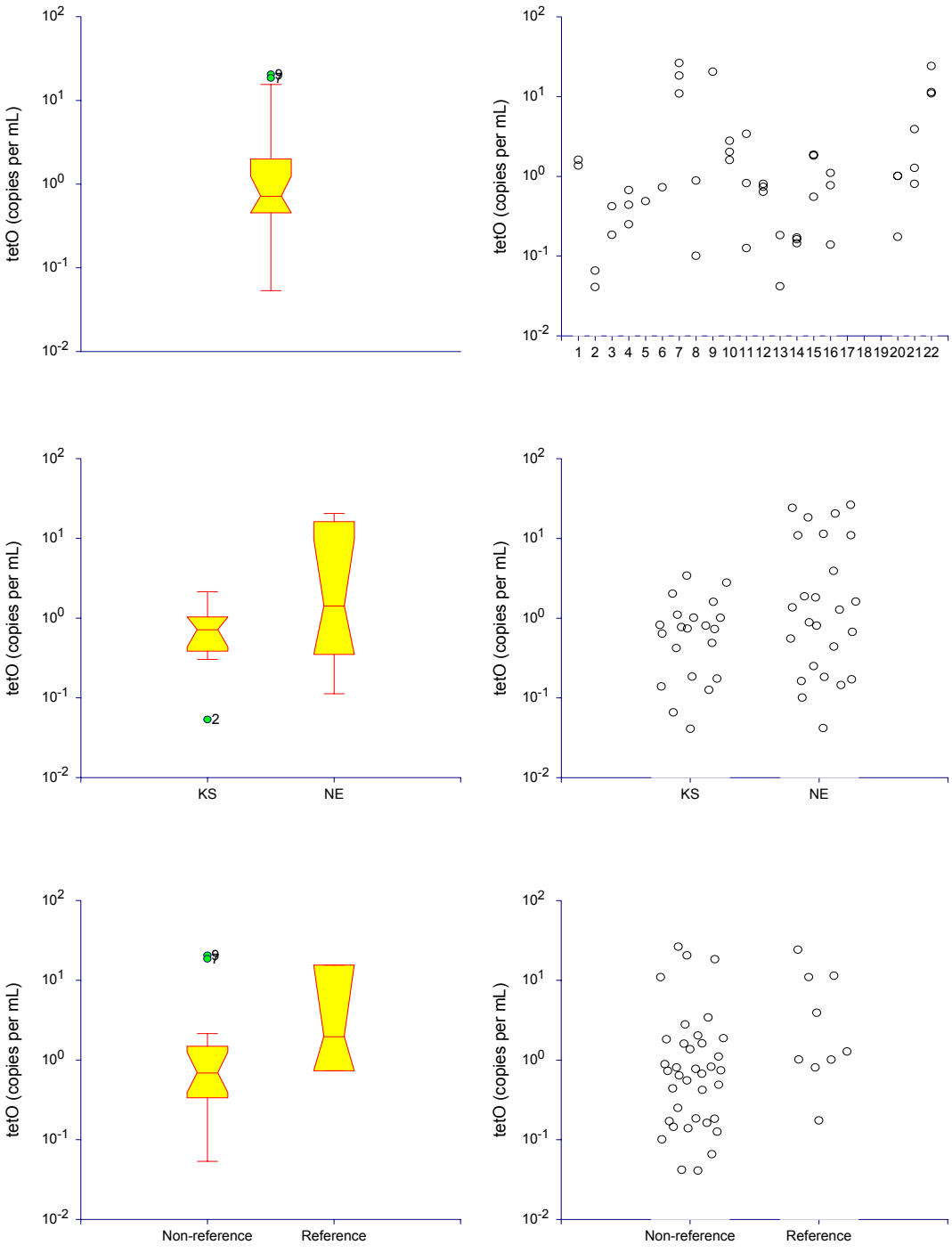

Figure E-1. Summary plots for tetO gene counts: box plots (a) for all measurements, (b) by state, and (c) by reference category; and dot plots (d) for all measurements, (e) by state, and (f) by reference category.

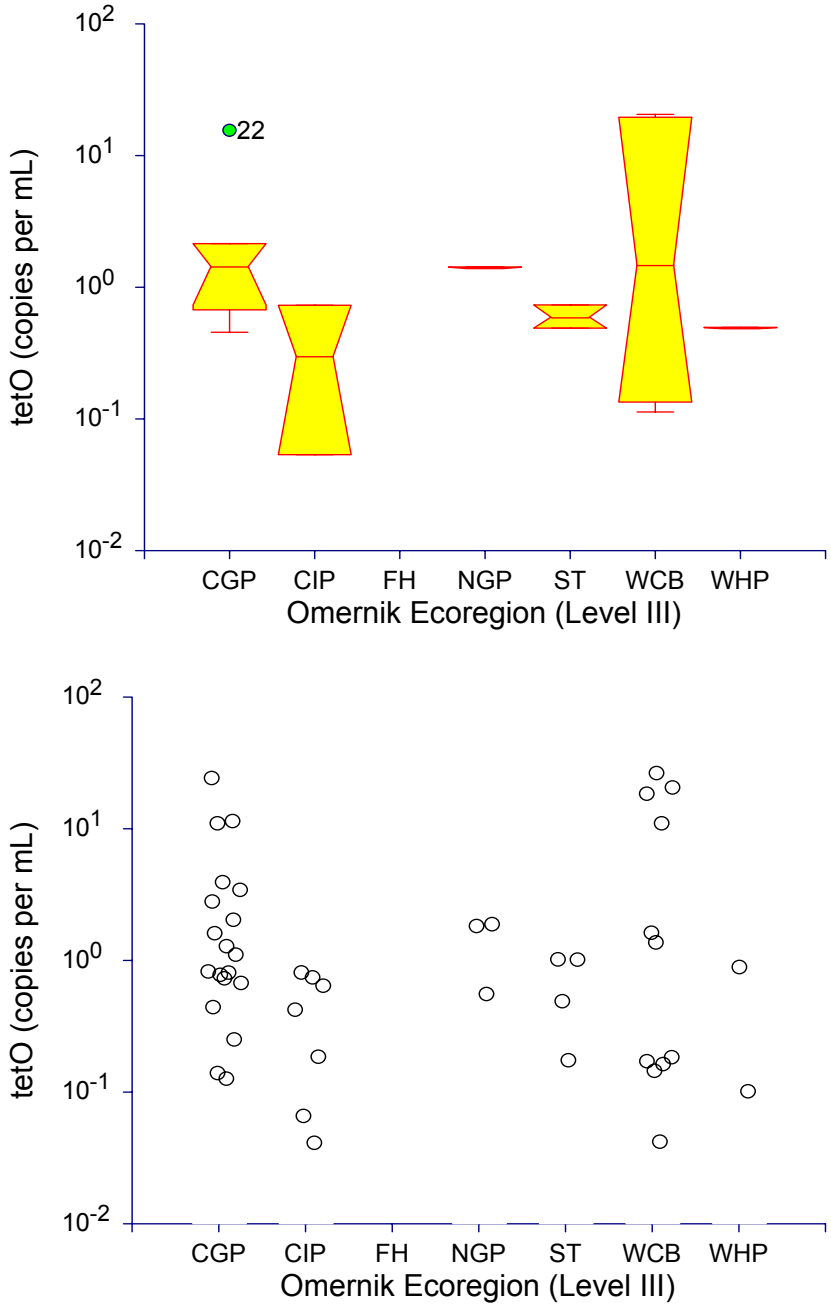

Figure E-2. Box plot and dot plot of tetO total gene counts by Omernik Level III Ecoregion. NGP: Northern Great Plains; WHP: Western High Plains; NSH: Northern Sand Hills; NGL: Northern Glaciated Plains; WCB: Western Corn Belt Plains; CGP: Central Great Plains; ST: Southwest Tablelands; COT: Central Oklahoma Tablelands; CIP: Central Irregular Plains; OH: Ozark Highlands.

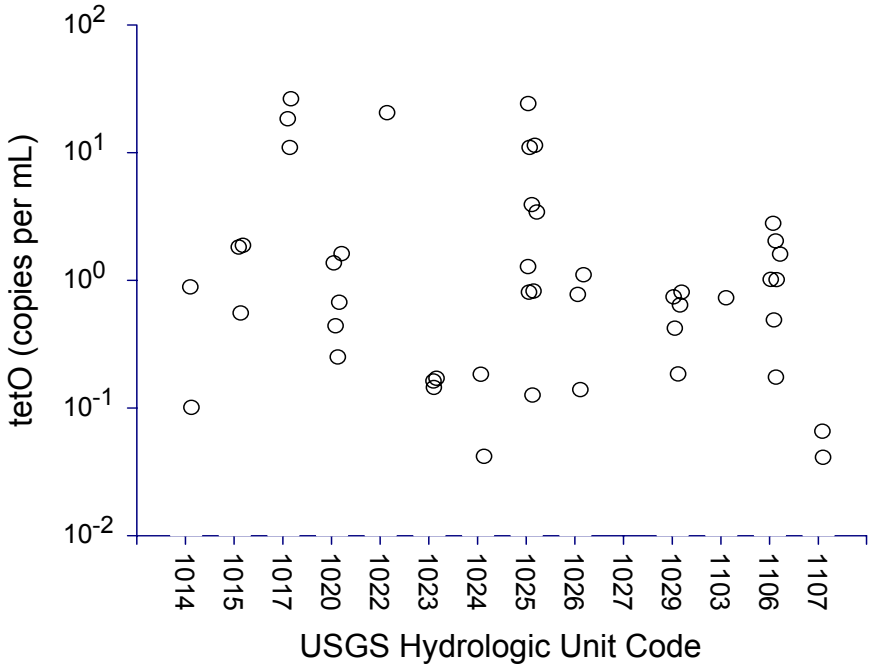

Figure E-3. Dot plot of tetO gene counts by 4 digit USGS Hydrologic Unit Code.

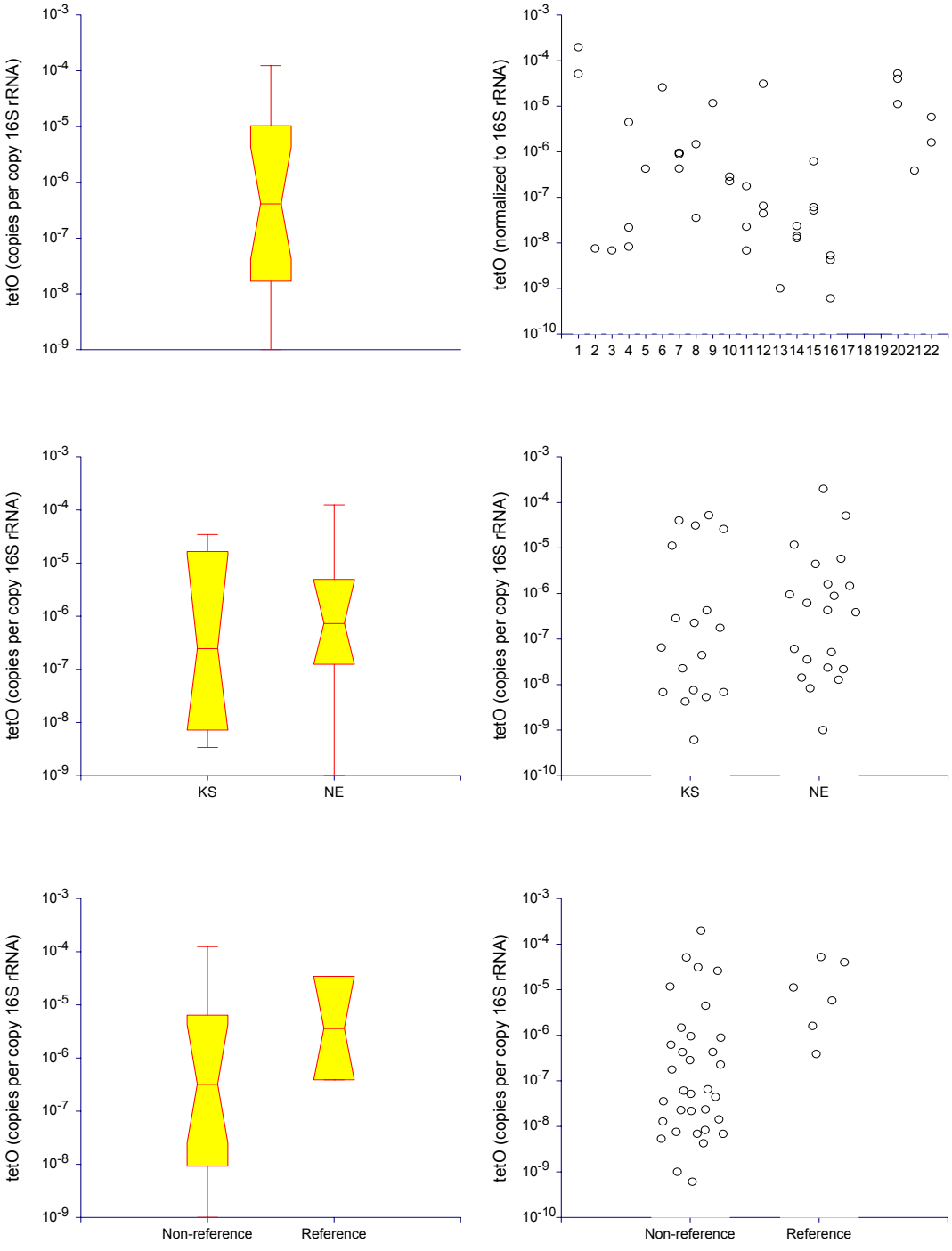

Figure E-4. Summary plots for tetO gene counts relative to 16SrRNA: box plots (a) for all measurements, (b) by state, and (c) by reference category; and dot plots (d) for all measurements, (e) by state, and (f) by reference category.

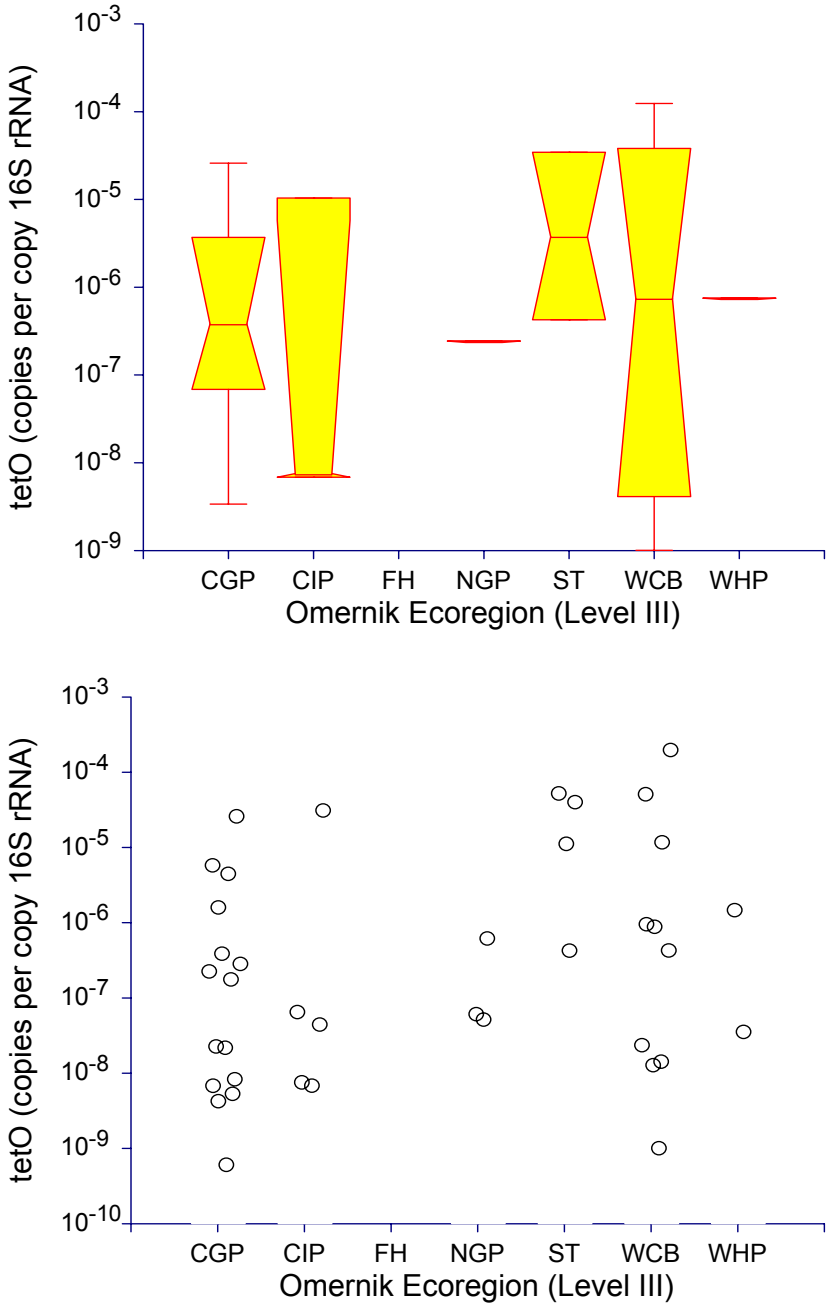

Figure E-5. Box plot and dot plot of tetO total gene counts relative to 16SrRNA by Omernik Level III Ecoregion. NGP: Northern Great Plains; WHP: Western High Plains; NSH: Northern Sand Hills; NGL: Northern Glaciated Plains; WCB: Western Corn Belt Plains; CGP: Central Great Plains; ST: Southwest Tablelands; COT: Central Oklahoma Tablelands; CIP: Central Irregular Plains; OH: Ozark Highlands.

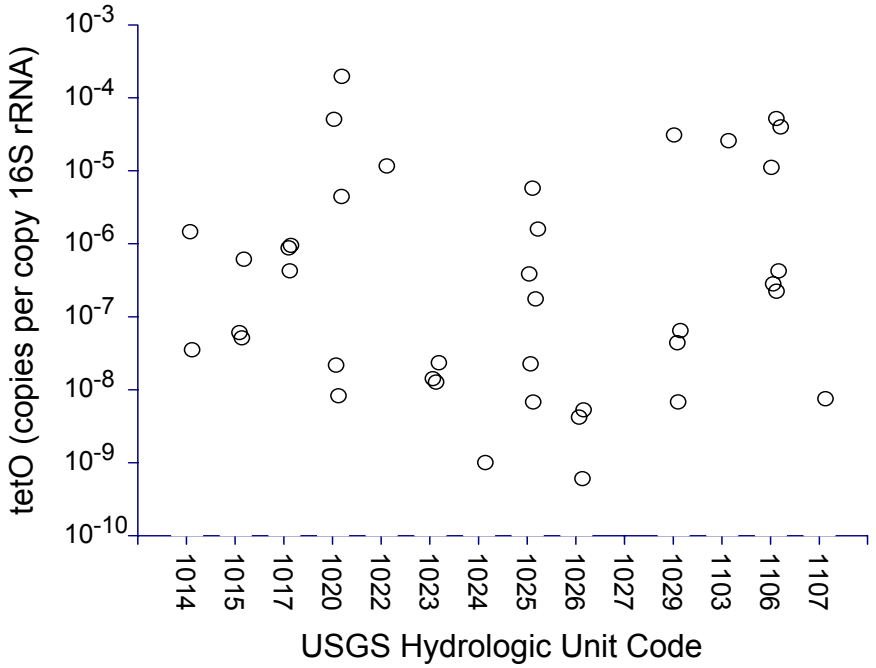

Figure E-6. Dot plot of tetO gene counts relative to 16SrRNA by 4 digit USGS Hydrologic Unit Code.

Appendix F – Predicted Values Tables

Cumulative distributions were calculated as described in the Predicted Values and Spatial Extents section of this report. For the tables in this Appendix:

Pct: Percentile
NResp: Number of observations used for estimate at this percentile
Estimate: Value of estimate provided by cumulative distribution
StdError: Standard error of the estimate
LCB95Pct: 95% Lower confidence bound of the estimate
UCB95Pct: 95% Upper confidence bound of the estimate

Tetracyclines

Table F-1. Predicted distributions of tetracyclines (parts per trillion) in perennial, wadeable streams of Kansas and Nebraska.

Statistic	NResp	Estimate	StdError	LCB95Pct	UCB95Pct
5Pct	1	82.9		82.9	131
10Pct	1	113		82.9	148
25Pct	4	180		82.9	243
50Pct	8	245		182	284
75Pct	12	287		249	436
90Pct	15	348		289	548
95Pct	16	411		326	548
Mean	17	255	24.3	207	302
Variance	17	10818	4447	2103	19533
Std. Deviation	17	104	21.4	62.1	146

Total Gene Counts

Table F-2. Predicted distributions of 16S-rRNA counts (copies per mL) in perennial, wadeable streams of Kansas and Nebraska. "Max" estimates were based on maximum of observed value for each site. "Avg" estimates were based on average of observed value for each site. "Min" estimates were based on maximum of observed value for each site.

Indicator	Statistic	NResp	Estimate	StdError	LCB95Pct	UCB95Pct
MaxOf16SrRNA	5Pct	1	2.559E+04		2.512E+04	2.665E+04
	10Pct	1	2.663E+04		2.550E+04	1.313E+06
	25Pct	4	3.013E+06		2.612E+04	9.908E+06
	50Pct	7	1.132E+07		3.114E+06	3.010E+07
	75Pct	12	3.017E+07		1.589E+07	5.871E+07
	90Pct	15	4.428E+07		3.016E+07	2.301E+08
	95Pct	15	5.634E+07		3.519E+07	2.301E+08
	Mean	17	2.564E+07	7.120E+06	1.169E+07	3.960E+07
	Variance	17	1.506E+15	1.066E+15	0.000E+00	3.595E+15
	Std. Deviation	17	3.881E+07	1.373E+07	1.190E+07	6.572E+07
AvgOf16SrRNA	5Pct	1	1.597E+04		1.597E+04	1.214E+06
	10Pct	1	2.153E+04		1.597E+04	1.938E+06
	25Pct	4	2.026E+06		1.597E+04	8.779E+06
	50Pct	8	9.966E+06		2.137E+06	1.909E+07
	75Pct	12	2.067E+07		1.001E+07	3.942E+07
	90Pct	15	3.253E+07		2.381E+07	2.071E+08
	95Pct	15	3.883E+07		2.752E+07	2.071E+08
	Mean	17	1.886E+07	5.844E+06	7.404E+06	3.031E+07
	Variance	17	1.157E+15	9.008E+14	0.000E+00	2.923E+15
	Std. Deviation	17	3.401E+07	1.324E+07	8.061E+06	5.997E+07
MinOf16SrRNA	5Pct	1	8.202E+03		8.202E+03	2.757E+04
	10Pct	1	1.230E+04		8.202E+03	1.113E+05
	25Pct	4	1.230E+05		8.202E+03	1.478E+06
	50Pct	8	2.412E+06		1.280E+05	9.439E+06
	75Pct	12	9.555E+06		4.778E+06	3.854E+07
	90Pct	15	2.898E+07		1.024E+07	1.832E+08
	95Pct	15	3.773E+07		1.241E+07	1.832E+08
	Mean	17	1.320E+07	5.249E+06	2.908E+06	2.348E+07
	Variance	17	9.563E+14	7.348E+14	0.000E+00	2.396E+15

tetW

Table F-3. Predicted distributions of tetW genes (copies per mL) in perennial, wadeable streams of Kansas. "Max" estimates were based on maximum of observed value for each site. "Avg" estimates were based on average of observed value for each site. "Min" estimates were based on maximum of observed value for each site.

Population	Indicator	Statistic	NResp	Estimate	StdError	LCB95Pct	UCB95Pct
Kansas							
	Max tetW	5Pct	1	0.106		0.106	0.646
		10Pct	1	0.209		0.106	0.921
		25Pct	2	0.957		0.106	1.99
		50Pct	4	2.03		0.606	9.38
		75Pct	6	5.25		1.53	23.1
		90Pct	8	16.2		4.72	23.1
		95Pct	8	19.6		5.56	23.1
		Mean	9	6.10	2.30	1.59	10.6
		Variance	9	55.7	25.7	5.41	106
		Std. Deviation	9	7.5	1.7	4.09	10.8
	Avg tetW	5Pct	1	0.106		0.106	0.390
		10Pct	1	0.160		0.106	0.505
		25Pct	2	0.520		0.106	1.29
		50Pct	4	1.30		0.439	4.97
		75Pct	6	4.21		1.15	15.8
		90Pct	8	7.79		3.57	15.8
		95Pct	8	11.8		4.60	15.8
		Mean	9	3.81	1.46	0.944	6.67
		Variance	9	22.6	12.7	0	47.5
		Std. Deviation	9	4.76	1.34	2.14	7.38
	Min tetW	5Pct	1	0.046		0.046	0.116
		10Pct	1	0.046		0.046	0.129
		25Pct	2	0.123		0.046	0.377
		50Pct	4	0.435		0.106	2.39
		75Pct	7	1.71		0.355	10.6
		90Pct	8	3.93		1.58	10.6
		95Pct	8	7.28		1.90	10.6
		Mean	9	2.09	0.952	0.228	3.96
		Variance	9	10.2	6.58	0	23.1
		Std. Deviation	9	3.20	1.03	1.18	5.22

Table F-4. Predicted distributions of relative proportion of tetW genes (copies per copy 16S-rRNA) in perennial, wadeable streams of Kansas. "Max" estimates were based on maximum of observed value for each site. "Avg" estimates were based on average of observed value for each site. "Min" estimates were based on maximum of observed value for each site.

Population	Indicator	Statistic	NResp	Estimate	StdError	LCB95Pct	UCB95Pct
Kansas							
	Max tetW	5Pct	1	1.56E-08		1.56E-08	4.55E-08
		10Pct	1	1.92E-08		1.56E-08	5.90E-08
		25Pct	2	5.70E-08		1.56E-08	8.80E-07
		50Pct	4	8.68E-07		4.23E-08	1.73E-05
		75Pct	6	7.12E-06		5.18E-07	2.75E-04
		90Pct	7	1.16E-04		1.37E-06	2.75E-04
		95Pct	7	1.95E-04		1.64E-06	2.75E-04
		Mean	8	4.68E-05	3.72E-05	-2.61E-05	1.20E-04
		Variance	8	9.83E-09	6.86E-09	0	2.33E-08
		Std. Deviation	8	9.91E-05	3.46E-05	3.13E-05	1.67E-04
	Avg tetW	5Pct	1	8.85E-09		8.85E-09	4.41E-08
		10Pct	1	1.33E-08		8.85E-09	4.77E-08
		25Pct	2	4.72E-08		8.85E-09	5.17E-07
		50Pct	4	4.93E-07		4.20E-08	8.86E-06
		75Pct	6	4.12E-06		2.20E-07	9.19E-05
		90Pct	7	4.11E-05		1.32E-06	9.19E-05
		95Pct	7	6.65E-05		1.51E-06	9.19E-05
		Mean	8	1.63E-05	1.24E-05	-7.92E-06	4.06E-05
		Variance	8	1.08E-09	7.50E-10	0	2.55E-09
		Std. Deviation	8	3.29E-05	1.14E-05	1.06E-05	5.53E-05
	Min tetW	5Pct	1	8.59E-10		8.59E-10	1.91E-08
		10Pct	1	3.06E-09		8.59E-10	2.75E-08
		25Pct	2	2.62E-08		8.59E-10	6.99E-08
		50Pct	4	5.89E-08		1.59E-08	6.78E-07
		75Pct	5	1.67E-07		4.87E-08	1.49E-06
		90Pct	7	1.25E-06		1.42E-07	1.49E-06
		95Pct	7	1.37E-06		1.68E-07	1.49E-06
		Mean	8	3.80E-07	1.37E-07	1.11E-07	6.50E-07
		Variance	8	3.07E-13	1.02E-13	1.08E-13	5.07E-13
		Std. Deviation	8	5.54E-07	9.17E-08	3.75E-07	7.34E-07

Table F-5. Predicted distributions of tetW genes (copies per mL) in perennial, wadeable streams of Nebraska. "Max" estimates were based on maximum of observed value for each site. "Avg" estimates were based on average of observed value for each site. "Min" estimates were based on maximum of observed value for each site.

Population	Indicator	Statistic	NResp	Estimate	StdError	LCB95Pct	UCB95Pct
Nebraska							
	Max tetW	5Pct	1	1.08		1.08	1.64
		10Pct	1	1.08		1.08	1.79
		25Pct	1	1.33		1.08	7.02
		50Pct	3	5.90		1.10	109
		75Pct	6	48.8		4.60	285
		90Pct	7	183		20.1	285
		95Pct	7	234		39.7	285
		Mean	8	62.7	32.1	-0.122	126
		Variance	8	9705	5069	0	19641
		Std. Deviation	8	98.5	25.7	48.1	149
	Avg tetW	5Pct	1	0.950		0.950	1.19
		10Pct	1	0.950		0.950	1.26
		25Pct	1	1.06		0.950	5.12
		50Pct	3	4.27		0.960	101
		75Pct	6	30.2		3.32	193
		90Pct	7	162		13.7	193
		95Pct	7	177		22.6	193
		Mean	8	48.7	24.2	1.15	96.2
		Variance	8	5468	2146	1262	9674
		Std. Deviation	8	73.9	14.5	45.5	102
	Min tetW	5Pct	1	0.444		0.444	0.647
		10Pct	1	0.444		0.444	0.738
		25Pct	1	0.638		0.444	2.06
		50Pct	3	2.72		0.527	80.9
		75Pct	6	15.5		2.18	154
		90Pct	7	135		8.77	154
		95Pct	7	145		9.84	154
		Mean	8	38.9	20.2	-0.644	78.5
		Variance	8	3685	1461	822	6548
		Std. Deviation	8	60.7	12.0	37.1	84.3

Table F-6. Predicted distributions of relative proportion of tetW genes (copies per copy 16S-rRNA) in perennial, wadeable streams of Nebraska. "Max" estimates were based on maximum of observed value for each site. "Avg" estimates were based on average of observed value for each site. "Min" estimates were based on maximum of observed value for each site.

Population	Indicator	Statistic	NResp	Estimate	StdError	LCB95Pct	UCB95Pct
Nebraska							
	Max tetW	5Pct	1	1.06E-08		1.06E-08	1.13E-07
		10Pct	1	1.06E-08		1.06E-08	5.44E-07
		25Pct	1	1.08E-07		1.06E-08	6.77E-06
		50Pct	4	7.23E-06		3.38E-08	7.04E-05
		75Pct	6	1.48E-05		6.73E-06	1.03E-03
		90Pct	7	2.92E-04		1.09E-05	1.03E-03
		95Pct	7	6.61E-04		1.13E-05	1.03E-03
		Mean	8	1.47E-04	1.17E-04	-8.29E-05	3.77E-04
		Variance	8	1.15E-07	8.78E-08	0	2.87E-07
		Std. Deviation	8	3.39E-04	1.29E-04	8.53E-05	5.93E-04
	Avg tetW	5Pct	1	1.06E-08		1.06E-08	7.73E-08
		10Pct	1	1.06E-08		1.06E-08	2.31E-07
		25Pct	1	7.45E-08		1.06E-08	2.75E-06
		50Pct	4	3.22E-06		2.58E-08	6.96E-05
		75Pct	6	1.13E-05		2.70E-06	6.77E-04
		90Pct	7	2.16E-04		6.99E-06	6.77E-04
		95Pct	7	4.46E-04		7.59E-06	6.77E-04
		Mean	8	1.00E-04	7.69E-05	-5.07E-05	2.51E-04
		Variance	8	4.95E-08	3.74E-08	0	1.23E-07
		Std. Deviation	8	2.23E-04	8.41E-05	5.78E-05	3.87E-04
	Min tetW	5Pct	1	1.06E-08		1.06E-08	4.82E-08
		10Pct	1	1.06E-08		1.06E-08	5.34E-08
		25Pct	2	4.76E-08		1.06E-08	9.93E-07
		50Pct	4	9.74E-07		2.08E-08	6.88E-05
		75Pct	6	8.20E-06		6.13E-07	3.33E-04
		90Pct	7	1.41E-04		2.69E-06	3.33E-04
		95Pct	7	2.37E-04		4.09E-06	3.33E-04
		Mean	8	5.46E-05	3.81E-05	-2.00E-05	1.29E-04
		Variance	8	1.22E-08	8.63E-09	0	2.91E-08
		Std. Deviation	8	1.10E-04	3.91E-05	3.36E-05	1.87E-04

tetQ

Table F-7. Predicted distributions of tetQ genes (copies per mL) in perennial, wadeable streams of Kansas and Nebraska. "Max" estimates were based on maximum of observed value for each site. "Avg" estimates were based on average of observed value for each site. "Min" estimates were based on maximum of observed value for each site.

Indicator	Statistic	NResp	Estimate	StdError	LCB95Pct	UCB95Pct
MaxOftetQ	5Pct	1	1.63		1.62	1.76
	10Pct	2	1.70		1.62	2.88
	25Pct	4	3.38		1.67	4.53
	50Pct	7	6.10		3.38	22.7
	75Pct	11	22.7		6.58	472
	90Pct	13	130		21.3	559
	95Pct	14	335		24.8	559
	Mean	15	69.2	45.3	-19.5	158
	Variance	15	25733	19348	0	63654
	Std. Deviation	15	160	60.3	42.2	279
AvgOftetQ	5Pct	1	0.945		0.945	1.62
	10Pct	2	1.57		0.945	1.76
	25Pct	4	1.98		1.18	3.63
	50Pct	7	5.26		1.99	14.9
	75Pct	11	15.1		5.64	260
	90Pct	13	71.3		13.8	307
	95Pct	14	185		17.1	307
	Mean	15	38.9	24.7	-9.52	87.4
	Variance	15	7716	5815	0	19113
	Std. Deviation	15	87.8	33.1	23.0	153
MinOftetQ	5Pct	1	0.0668		0.0668	0.719
	10Pct	1	0.0849		0.0668	1.25
	25Pct	3	1.19		0.0668	2.32
	50Pct	7	2.92		1.47	6.14
	75Pct	11	6.77		3.11	69.0
	90Pct	13	26.0		6.38	74.9
	95Pct	14	50.4		7.46	74.9
	Mean	15	11.4	5.88	-0.135	22.9
	Variance	15	452	326	0	1091
	Std. Deviation	15	21.3	7.67	6.21	36.3

Table F-8. Predicted distributions of relative proportion of tetQ genes (copies per copy 16S-rRNA) in perennial, wadeable streams of Kansas and Nebraska. "Max" estimates were based on maximum of observed value for each site. "Avg" estimates were based on average of observed value for each site. "Min" estimates were based on maximum of observed value for each site.

Indicator	Statistic	NResp	Estimate	StdError	LCB95Pct	UCB95Pct
MaxOftetQ	5Pct	1	1.60E-09		1.60E-09	6.89E-08
	10Pct	1	8.37E-09		1.60E-09	2.84E-07
	25Pct	4	2.44E-07		1.60E-09	1.67E-06
	50Pct	7	4.77E-06		2.01E-07	2.78E-05
	75Pct	11	2.67E-05		5.66E-06	2.27E-03
	90Pct	13	3.11E-04		2.12E-05	2.52E-03
	95Pct	14	1.36E-03		3.26E-05	2.52E-03
	Mean	15	2.65E-04	2.07E-04	-1.41E-04	6.71E-04
	Variance	15	5.32E-07	4.13E-07	0	1.34E-06
	Std. Deviation	15	7.29E-04	2.84E-04	1.74E-04	1.28E-03
AvgOftetQ	5Pct	1	1.60E-09		1.60E-09	5.64E-08
	10Pct	1	3.82E-09		1.60E-09	2.18E-07
	25Pct	4	1.88E-07		1.60E-09	7.85E-07
	50Pct	7	4.67E-06		1.57E-07	1.42E-05
	75Pct	11	1.35E-05		5.16E-06	1.46E-03
	90Pct	13	1.09E-04		1.02E-05	1.63E-03
	95Pct	14	8.22E-04		1.71E-05	1.63E-03
	Mean	15	1.66E-04	1.34E-04	-9.68E-05	4.28E-04
	Variance	15	2.22E-07	1.75E-07	0	5.65E-07
	Std. Deviation	15	4.72E-04	1.85E-04	1.09E-04	8.34E-04
MinOftetQ	5Pct	1	8.64E-10		6.75E-10	1.43E-09
	10Pct	1	1.36E-09		7.61E-10	1.50E-08
	25Pct	3	5.89E-08		1.04E-09	2.45E-07
	50Pct	7	2.58E-07		6.10E-08	4.12E-06
	75Pct	11	4.49E-06		2.62E-07	2.69E-04
	90Pct	13	5.03E-05		3.84E-06	3.27E-04
	95Pct	14	1.83E-04		5.08E-06	3.27E-04
	Mean	15	3.44E-05	2.70E-05	-1.84E-05	8.73E-05
	Variance	15	8.99E-09	6.96E-09	0	2.26E-08
	Std. Deviation	15	9.48E-05	3.67E-05	2.28E-05	1.67E-04

tetO

Table F-9. Predicted distributions of tetO genes (copies per mL) in perennial, wadeable streams of Kansas and Nebraska. "Max" estimates were based on maximum of observed value for each site. "Avg" estimates were based on average of observed value for each site. "Min" estimates were based on maximum of observed value for each site.

Indicator	Statistic	NResp	Estimate	StdError	LCB95Pct	UCB95Pct
MaxOftetO	5Pct	1	0.0660		0.0660	0.139
	10Pct	1	0.109		0.0660	0.173
	25Pct	2	0.184		0.0674	0.746
	50Pct	8	0.820		0.292	1.80
	75Pct	12	1.92		0.858	23.7
	90Pct	14	17.7		1.73	26.5
	95Pct	15	23.0		3.03	26.5
	Mean	16	4.83	2.19	0.538	9.13
	Variance	16	74.4	34.6	6.57	142
	Std. Deviation	16	8.63	2.01	4.69	12.6
AvgOftetO	5Pct	1	0.0535		0.0535	0.110
	10Pct	1	0.0868		0.0535	0.131
	25Pct	2	0.158		0.0855	0.490
	50Pct	7	0.609		0.158	1.48
	75Pct	12	1.48		0.704	19.9
	90Pct	14	15.9		1.46	20.6
	95Pct	15	19.4		1.63	20.6
	Mean	16	3.94	1.83	0.362	7.52
	Variance	16	51.3	23.0	6.28	96.3
	Std. Deviation	16	7.16	1.60	4.02	10.3
MinOftetO	5Pct	1	0.0411		0.0411	0.0419
	10Pct	1	0.0416		0.0411	0.0732
	25Pct	3	0.118		0.0411	0.190
	50Pct	7	0.241		0.121	1.05
	75Pct	12	1.08		0.377	17.1
	90Pct	14	9.42		0.809	20.6
	95Pct	15	15.0		1.42	20.6
	Mean	16	3.07	1.60	-0.0744	6.21
	Variance	16	37.6	22.9	0	82.5
	Std. Deviation	16	6.13	1.87	2.48	9.79

Table F-10. Predicted distributions of relative proportion of tetO genes (copies per copy 16S-rRNA) in perennial, wadeable streams of Kansas and Nebraska. "Max" estimates were based on maximum of observed value for each site. "Avg" estimates were based on average of observed value for each site. "Min" estimates were based on maximum of observed value for each site.

Indicator	Statistic	NResp	Estimate	StdError	LCB95Pct	UCB95Pct
MaxOftetO	5Pct	1	1.01E-09		1.01E-09	7.35E-09
	10Pct	1	3.20E-09		1.01E-09	1.21E-08
	25Pct	4	1.41E-08		2.30E-09	3.09E-07
	50Pct	9	6.22E-07		1.44E-08	5.40E-06
	75Pct	12	5.60E-06		7.32E-07	1.43E-04
	90Pct	14	2.97E-05		4.15E-06	1.98E-04
	95Pct	15	1.01E-04		1.01E-05	1.98E-04
	Mean	16	2.12E-05	1.48E-05	-7.80E-06	5.02E-05
	Variance	16	3.00E-09	2.32E-09	0	7.55E-09
	Std. Deviation	16	5.48E-05	2.12E-05	1.32E-05	9.63E-05
AvgOftetO	5Pct	1	1.01E-09		1.01E-09	7.35E-09
	10Pct	1	2.22E-09		1.01E-09	1.02E-08
	25Pct	4	1.14E-08		1.72E-09	2.06E-07
	50Pct	9	4.29E-07		1.16E-08	3.40E-06
	75Pct	12	3.81E-06		5.36E-07	9.19E-05
	90Pct	14	2.09E-05		1.49E-06	1.24E-04
	95Pct	15	6.68E-05		1.06E-05	1.24E-04
	Mean	16	1.35E-05	9.26E-06	-4.66E-06	3.16E-05
	Variance	16	1.18E-09	9.07E-10	0	2.96E-09
	Std. Deviation	16	3.44E-05	1.32E-05	8.47E-06	6.02E-05
MinOftetO	5Pct	1	7.10E-10		6.07E-10	9.57E-10
	10Pct	1	9.45E-10		6.85E-10	6.81E-09
	25Pct	5	7.64E-09		9.08E-10	1.21E-08
	50Pct	7	3.27E-08		7.83E-09	4.27E-07
	75Pct	12	4.27E-07		3.85E-08	4.26E-05
	90Pct	14	2.09E-05		4.27E-07	5.10E-05
	95Pct	15	3.64E-05		2.34E-06	5.10E-05
	Mean	16	6.49E-06	3.92E-06	-1.20E-06	1.42E-05
	Variance	16	2.20E-10	1.47E-10	0	5.07E-10
	Std. Deviation	16	1.48E-05	4.95E-06	5.12E-06	2.45E-05

Appendix G – Contextual Values Of Tetracyclines (Raw Data)

Table G-1. Contextual values raw data. Values were converted to parts per trillion (ppt) for comparison, and disturbance codes were assigned based on station descriptions in the original text. In the case where data points are detection limits, the detection limit value was taken as a conservative estimate of the compound. Source 1: (Campagnolo et al. 2002); Source 2: this study; Source 3: (Hirsch et al. 1999); Source 4: (Kolpin et al. 2002); Source 5: (Mackie et al. 2006); Source 6: (Pei et al. 2006). (HI) "Heavily Impacted" – by urban areas and/or agriculture, such as sewage treatment effluents, feedlot waste lagoons, feedlot soils, raw manure, and areas noted as both urban and agricultural by the authors; (LU) "Lightly Impacted Urban" – urban areas other than sewage treatment outfalls; (LA) "Lightly Impacted Agriculture" – areas such as pasture land, crop land, streams and rivers in croplands or pastures, irrigation ditches, and any sites with otherwise unidentifiable disturbance conditions; and (P) "Pristine" – areas specifically identified by the studies' authors as pristine or reference condition sites. Sites from this study are labeled as disturbance category (X) "Experimental." Tetracycline types are ctc: chlortetracycline; tc: tetracycline; otc: oxytetracycline; totTCs: sum of tetracyclines as a class; dxc: doxycycline; dmc: demeclocycline; mcc: meclocycline. If reported values were measured at or below detection limits, then they are indicated by an X. All values have been converted to parts per trillion (ppt).

Source	Disturbance Code	Station	Tetracycline Type	Detect Limit	Value (ppt)
1	HI	swine field tile - LC/EMI-MS	ctc	X	500
1	HI	swine field tile - LC/EMI-MS	ctc		2000
1	HI	swine lagoon - LC/EMI-MS	ctc		8.7E+05
1	HI	swine lagoon - LC/EMI-MS	ctc		6.8E+04
1	HI	swine lagoon - LC/EMI-MS	ctc		9.5E+04
1	HI	swine lagoon - LC/EMI-MS	ctc		1.9E+05
1	HI	swine lagoon - LC/EMI-MS	ctc		2.5E+05
1	HI	swine lagoon - LC/EMI-MS	ctc		1.0E+06
1	HI	swine lagoon - LC/EMI-MS	ctc		7.0E+04
1	HI	swine lagoon - assay	tc		2.5E+05
1	HI	swine lagoon - assay	tc		1.1E+04
1	HI	swine lagoon - assay	tc		1.5E+05
1	HI	swine lagoon - assay	tc		6.8E+04
1	HI	swine lagoon - assay	tc		6.6E+04
1	HI	swine lagoon - assay	tc		5.4E+05
1	HI	swine lagoon - assay	tc		1.1E+05
1	HI	swine field tile - LC/EMI-MS	tcAndOtc	X	500
1	HI	swine field tile - LC/EMI-MS	tcAndOtc	X	500
1	HI	swine lagoon - LC/EMI-MS	tcAndOtc		1.3E+05

Source	Disturbance Code	Station	Tetracycline Type	Detect Limit	Value (ppt)
1	HI	swine lagoon - LC/EMI-MS	tcAndOtc		2.7E+04
1	HI	swine lagoon - LC/EMI-MS	tcAndOtc		3.5E+04
1	HI	swine lagoon - LC/EMI-MS	tcAndOtc		2.5E+04
1	HI	swine lagoon - LC/EMI-MS	tcAndOtc		1.0E+05
1	HI	swine lagoon - LC/EMI-MS	tcAndOtc		4.1E+05
1	HI	swine lagoon - LC/EMI-MS	tcAndOtc		2.5E+04
1	LA	poultry farm spring - LC/EMI-MS	ctc	X	500
1	LA	poultry farm spring - LC/EMI-MS	ctc	X	500
1	LA	poultry field stream - LC/EMI-MS	ctc	X	500
1	LA	poultry field stream - LC/EMI-MS	ctc		1500
1	LA	poultry field stream - LC/EMI-MS	ctc	X	500
1	LA	poultry field stream - LC/EMI-MS	ctc	X	500
1	LA	poultry field stream - LC/EMI-MS	ctc	X	500
1	LA	poultry field stream - LC/EMI-MS	ctc	X	500
1	LA	poultry field stream - LC/EMI-MS	ctc	X	500
1	LA	poultry field tile - LC/EMI-MS	ctc	X	500
1	LA	poultry field well - LC/EMI-MS	ctc	X	500
1	LA	poultry river - LC/EMI-MS	ctc	X	500
1	LA	swine monitoring well - LC/EMI-MS	ctc	X	500
1	LA	swine monitoring well - LC/EMI-MS	ctc	X	500
1	LA	swine monitoring well - LC/EMI-MS	ctc	X	500
1	LA	poultry river - assay	tc	X	1000
1	LA	swine monitoring well - assay	tc	X	1000
1	LA	poultry farm spring - LC/EMI-MS	tcAndOtc		2000
1	LA	poultry farm spring - LC/EMI-MS	tcAndOtc		1000
1	LA	poultry field stream - LC/EMI-MS	tcAndOtc	X	500
1	LA	poultry field stream - LC/EMI-MS	tcAndOtc	X	500
1	LA	poultry field stream - LC/EMI-MS	tcAndOtc		1000

Source	Disturbance Code	Station	Tetracycline Type	Detect Limit	Value (ppt)
1	LA	poultry field stream - LC/EMI-MS	tcAndOtc	X	500
1	LA	poultry field stream - LC/EMI-MS	tcAndOtc	X	500
1	LA	poultry field stream - LC/EMI-MS	tcAndOtc	X	500
1	LA	poultry field stream - LC/EMI-MS	tcAndOtc	X	500
1	LA	poultry field tile - LC/EMI-MS	tcAndOtc	X	500
1	LA	poultry field well - LC/EMI-MS	tcAndOtc		1000
1	LA	poultry river - LC/EMI-MS	tcAndOtc	X	500
1	LA	swine monitoring well - LC/EMI-MS	tcAndOtc	X	500
1	LA	swine monitoring well - LC/EMI-MS	tcAndOtc	X	500
1	LA	swine monitoring well - LC/EMI-MS	tcAndOtc	X	500
2	X	Site 1	totTCs		133.4
2	X	Site 2	totTCs		82.9
2	X	Site 3	totTCs		240.1
2	X	Site 4	totTCs		387.3
2	X	Site 5	totTCs		246.3
2	X	Site 6	totTCs		217.5
2	X	Site 7	totTCs		190
2	X	Site 8	totTCs		159.8
2	X	Site 9	totTCs		297.5
2	X	Site 10	totTCs		279.5
2	X	Site 11	totTCs		268
2	X	Site 12	totTCs		327
2	X	Site 13	totTCs		244.2
2	X	Site 14	totTCs		263.6
2	X	Site 15	totTCs		548.3
2	X	Site 16	totTCs		324.4
2	X	Site 17	totTCs		179.4
2	X	Site 18	totTCs		420.7
2	X	Site 19	totTCs		77.8
2	X	Site 20	totTCs		184.2
2	X	Site 21	totTCs		85.4
2	X	Site 22	totTCs		78.1
3	HI	sewage trtmt plant effluent	ctc	X	50
3	HI	sewage tetM plant effluent	dxc	X	50
3	HI	sewage tetM plant effluent	otc	X	50
3	HI	sewage tetM plant effluent	tc	X	50
3	LA	ground water	ctc	X	50
3	LA	surface water	ctc	X	50

Source	Disturbance Code	Station	Tetracycline Type	Detect Limit	Value (ppt)
3	LA	ground water	dxc	X	50
3	LA	surface water	dxc	X	50
3	LA	ground water	otc	X	50
3	LA	surface water	otc	X	50
3	LA	ground water	tc	X	50
3	LA	surface water	tc	X	50
4	LA	streams - max - LC/EMI-MS	ctc	X	50
4	LA	streams - max - LC/EMI-MS	ctc		690
4	LA	streams - median - LC/EMI-MS	ctc	X	50
4	LA	streams - median - LC/EMI-MS	ctc		420
4	LA	streams - max - LC/EMI-MS	dxc	X	100
4	LA	streams - median - LC/EMI-MS	dxc	X	100
4	LA	streams - max - LC/EMI-MS	otc	X	100
4	LA	streams - max - LC/EMI-MS	otc		340
4	LA	streams - median - LC/EMI-MS	otc	X	100
4	LA	streams - median - LC/EMI-MS	otc		340
4	LA	streams - max - LC/EMI-MS	tc	X	50
4	LA	streams - max - LC/EMI-MS	tc		110
4	LA	streams - median - LC/EMI-MS	tc	X	50
4	LA	streams - median - LC/EMI-MS	tc		110
5	HI	manure - Site A - LC/EMI-MS	ctc		100
5	HI	manure - Site A - LC/EMI-MS	ctc		1.4E+04
5	HI	manure - Site C - LC/EMI-MS	ctc		8900
5	HI	manure - Site C - LC/EMI-MS	ctc		1.3E+05
5	HI	manure - Site A - LC/EMI-MS	otc		350
5	HI	manure - Site A - LC/EMI-MS	otc		410
5	HI	manure - Site C - LC/EMI-MS	otc		4260
5	HI	manure - Site A - LC/EMI-MS	tc		400
5	HI	manure - Site A - LC/EMI-MS	tc		8200
5	HI	manure - Site C - LC/EMI-MS	tc		2600
5	HI	manure - Site C - LC/EMI-MS	tc		8500

Source	Disturbance Code	Station	Tetracycline Type	Detect Limit	Value (ppt)
5	LA	ground water near swine - Site A - LC/EMI-MS	otc		80
5	LA	ground water near swine - Site A - LC/EMI-MS	otc		130
5	LA	ground water near swine - Site C - LC/EMI-MS	tc		400
6	HI	site 4 - heavy agriculture - high flow	ctc		4600
6	HI	site 4 - heavy agriculture - low flow	ctc		1.6E+04
6	HI	site 5 - urban and agriculture - high flow	ctc		3800
6	HI	site 5 - urban and agriculture - low flow	ctc		2.2E+04
6	HI	site 4 - heavy agriculture - high flow	dmc		2100
6	HI	site 4 - heavy agriculture - low flow	dmc		9500
6	HI	site 5 - urban and agriculture - high flow	dmc		6900
6	HI	site 5 - urban and agriculture - low flow	dmc		2.4E+04
6	HI	site 4 - heavy agriculture - high flow	dxc		6300
6	HI	site 4 - heavy agriculture - low flow	dxc		1.2E+04
6	HI	site 5 - urban and agriculture - high flow	dxc		1.5E+04
6	HI	site 5 - urban and agriculture - low flow	dxc		2.6E+04
6	HI	site 4 - heavy agriculture - high flow	mcc		2.8E+04
6	HI	site 4 - heavy agriculture - low flow	mcc		2.6E+04
6	HI	site 5 - urban and agriculture - high flow	mcc		4.2E+04
6	HI	site 5 - urban and agriculture - low flow	mcc		7.2E+04
6	HI	site 4 - heavy agriculture - high flow	otc		7400
6	HI	site 4 - heavy agriculture - low flow	otc		1.9E+04
6	HI	site 5 - urban and agriculture - high flow	otc		2.4E+04
6	HI	site 5 - urban and agriculture - low flow	otc		3.6E+04
6	HI	site 4 - heavy agriculture - high flow	tc		8400
6	HI	site 4 - heavy agriculture - low flow	tc		3900

Source	Disturbance Code	Station	Tetracycline Type	Detect Limit	Value (ppt)
6	HI	site 5 - urban and agriculture - high flow	tc		1.0E+04
6	HI	site 5 - urban and agriculture - low flow	tc		2.5E+04
6	HI	site 4 - heavy agriculture - high flow	totTCs		5.7E+04
6	HI	site 4 - heavy agriculture - low flow	totTCs		8.6E+04
6	HI	site 5 - urban and agriculture - high flow	totTCs		1.0E+05
6	HI	site 5 - urban and agriculture - low flow	totTCs		2.0E+05
6	LU	site 3 - urban area - high flow	ctc		3100
6	LU	site 3 - urban area - low flow	ctc		1.9E+04
6	LU	site 3 - urban area - high flow	dmc		6800
6	LU	site 3 - urban area - low flow	dmc		1.5E+04
6	LU	site 3 - urban area - high flow	dxc		1.0E+04
6	LU	site 3 - urban area - low flow	dxc		1.3E+04
6	LU	site 3 - urban area - high flow	mcc		2.2E+04
6	LU	site 3 - urban area - low flow	mcc		1.7E+05
6	LU	site 3 - urban area - high flow	otc		7300
6	LU	site 3 - urban area - low flow	otc		5.6E+04
6	LU	site 3 - urban area - high flow	tc		8700
6	LU	site 3 - urban area - low flow	tc		1.0E+05
6	LU	site 3 - urban area - high flow	totTCs		5.8E+04
6	LU	site 3 - urban area - low flow	totTCs		8.7E+04
6	LA	site 2 - light agriculture - high flow	ctc		3000
6	LA	site 2 - light agriculture - low flow	ctc		9600
6	LA	site 2 - light agriculture - high flow	dmc		2100
6	LA	site 2 - light agriculture - low flow	dmc		6500
6	LA	site 2 - light agriculture - high flow	dxc		5100

Source	Disturbance Code	Station	Tetracycline Type	Detect Limit	Value (ppt)
6	LA	site 2 - light agriculture - low flow	dxc		1.3E+04
6	LA	site 2 - light agriculture - high flow	mcc		3.0E+04
6	LA	site 2 - light agriculture - low flow	mcc		3.8E+04
6	LA	site 2 - light agriculture - high flow	otc		2400
6	LA	site 2 - light agriculture - low flow	otc		7800
6	LA	site 2 - light agriculture - high flow	tc		3600
6	LA	site 2 - light agriculture - low flow	tc		1.1E+04
6	LA	site 2 - light agriculture - high flow	totTCs		4.6E+04
6	LA	site 2 - light agriculture - low flow	totTCs		8.7E+04
6	P	site 1 - pristine area - high flow	ctc	X	40
6	P	site 1 - pristine area - low flow	ctc	X	40
6	P	site 1 - pristine area - high flow	dmc	X	40
6	P	site 1 - pristine area - low flow	dmc	X	40
6	P	site 1 - pristine area - high flow	dxc	X	40
6	P	site 1 - pristine area - low flow	dxc	X	40
6	P	site 1 - pristine area - high flow	mcc	X	40
6	P	site 1 - pristine area - low flow	mcc	X	40
6	P	site 1 - pristine area - high flow	otc	X	40
6	P	site 1 - pristine area - low flow	otc	X	40
6	P	site 1 - pristine area - high flow	tc	X	40
6	P	site 1 - pristine area - low flow	tc	X	40
6	P	site 1 - pristine area - high flow	totTCs	X	40
6	P	site 1 - pristine area - low flow	totTCs	X	40

Appendix H – Contextual Values Of Tetracycline Resistance Genes (Raw Data)

Table H-1. Contextual values raw data. In the case where data points are detection limits, the detection limit value was taken as a conservative estimate of the compound. Source 1: (Auerbach et al. 2007); Source 2: this study; Source 3: (Hirsch et al. 1999); Source 4: (Kolpin et al. 2002); Source 5: (Mackie et al. 2006); Source 6: (Patterson et al. 2007); Source 7: (Pei et al. 2006), Source 8: (Pruden et al. 2006). (HI) "Heavily Impacted" – by urban areas and/or agriculture, such as sewage treatment effluents, feedlot waste lagoons, feedlot soils, raw manure, and areas noted as both urban and agricultural by the authors; (LU) "Lightly Impacted Urban" – urban areas other than sewage treatment outfalls; (LA) "Lightly Impacted Agriculture" – areas such as pasture land, crop land, streams and rivers in croplands or pastures, irrigation ditches, and any sites with otherwise unidentifiable disturbance conditions; and (P) "Pristine" – areas specifically identified by the studies' authors as pristine or reference condition sites. Sites from this study are labeled as disturbance category (X) "Experimental." Units for each observation were converted to the equivalent units shown (e.g., copies per copy 16S-rRNA = % of copies of 16S-rRNA * 100 and ug/L = 1 x 10^{-6} copies per copy).

Source	Disturbance Code	Station	tetW	tetQ	tetO	Units
1	HI	wwtp act sludge (jul)		0.01		copy per copy 16S
1	HI	wwtp act sludge (seasonal avg)		0.01		copy per copy 16S
1	HI	wwtp act sludge (seasonal avg)		0.1		copy per copy 16S
1	HI	wwtp biosolids (jul)		1.5		copy per copy 16S
1	HI	wwtp biosolids (seasonal avg)		0.01		copy per copy 16S
1	HI	wwtp biosolids (seasonal avg)		1.8		copy per copy 16S
1	HI	wwtp effluent (jul)		1.8		copy per copy 16S
1	HI	wwtp effluent (seasonal avg)		0.1		copy per copy 16S
1	HI	wwtp effluent (seasonal avg)		0.5		copy per copy 16S
1	HI	wwtp effluent w/o UV (apr)		0.42		copy per copy 16S

Source	Disturbance Code	Station	tetW	tetQ	tetO	Units
1	HI	wwtp effluent with UV (apr)		0.28		copy per copy 16S
1	HI	wwtp influent (jul)		1.1		copy per copy 16S
1	HI	wwtp influent (seasonal avg)		1.1		copy per copy 16S
1	HI	wwtp influent (seasonal avg)		6.2		copy per copy 16S
2	X	Site 1	0.000677	0.001631	0.000124	copy per copy 16S
2	X	Site 2	5.42E-08	4.7E-08	7.56E-09	copy per copy 16S
2	X	Site 3	4.36E-08	6.12E-08	6.84E-09	copy per copy 16S
2	X	Site 4	2.35E-06	7.03E-06	1.5E-06	copy per copy 16S
2	X	Site 5	1.2E-06	4.82E-06	4.26E-07	copy per copy 16S
2	X	Site 6	1.16E-05	5.77E-05	2.6E-05	copy per copy 16S
2	X	Site 7	7.82E-06	1E-05	7.56E-07	copy per copy 16S
2	X	Site 8	6.31E-06	1.87E-05	7.51E-07	copy per copy 16S
2	X	Site 9	8.77E-05		1.17E-05	copy per copy 16S
2	X	Site 10	1.69E-06	6.54E-06	2.54E-07	copy per copy 16S
2	X	Site 11	3.24E-07	1.48E-06	6.86E-08	copy per copy 16S
2	X	Site 12	9.19E-05	0.000115	1.04E-05	copy per copy 16S
2	X	Site 13	1.06E-08	1.6E-09	1.01E-09	copy per copy 16S
2	X	Site 14	1.01E-07	4.3E-07	1.69E-08	copy per copy 16S
2	X	Site 15	2.93E-06	3.55E-07	2.44E-07	copy per copy 16S
2	X	Site 16	8.85E-09	1.25E-08	3.39E-09	copy per copy 16S
2	X	Site 17				copy per copy 16S
2	X	Site 18	1.13E-08	6.27E-08		copy per copy 16S

Source	Disturbance Code	Station	tetW	tetQ	tetO	Units
2	X	Site 19				copy per copy 16S
2	X	Site 20	0.000222	0.000676	3.45E-05	copy per copy 16S
2	X	Site 21	2.37E-06	2.96E-06	3.88E-07	copy per copy 16S
2	X	Site 22	4.14E-05	0.00014	3.7E-06	copy per copy 16S
3	LA	ground water				ug/L
3	HI	sewage trt plant effluent				ug/L
3	LA	surface water				ug/L
4	LA	streams - max - LC/EMI-MS				ug/L
4	LA	streams - max - LC/EMI-MS				ug/L
4	LA	streams - median - LC/EMI-MS				ug/L
4	LA	streams - median - LC/EMI-MS				ug/L
5	HI	cattle lagoon site A (jul)		6.91		% of 16S*100
5	HI	cattle lagoon site A (seasonal range)		243		% of 16S*100
5	HI	cattle lagoon site A (seasonal range)		1301		% of 16S*100
5	HI	cattle lagoon site C (jul)		5		% of 16S*100
5	HI	cattle lagoon site C (seasonal range)		120		% of 16S*100
5	HI	cattle lagoon site C (seasonal range)		2103		% of 16S*100
5	LA	ground water near swine - Site A - LC/EMI-MS				ug/L
5	LA	ground water near swine - Site A - LC/EMI-MS				ug/L
5	LA	ground water near swine - Site C - LC/EMI-MS				ug/L
5	HI	manure - Site A - LC/EMI-MS				ug/L
5	HI	manure - Site A - LC/EMI-MS				ug/L

Source	Disturbance Code	Station	tetW	tetQ	tetO	Units
5	HI	manure - Site C - LC/EMI-MS				ug/L
5	HI	manure - Site C - LC/EMI-MS				ug/L
6	HI	English cow	40			% of 16S control * 100
6	HI	English pig	1330	200	240	% of 16S control * 100
6	HI	Italian pig 1	1960	1400	670	% of 16S control * 100
6	HI	Italian pig 2	1410	740	510	% of 16S control * 100
6	HI	Norway pig herd 1	390	120	240	% of 16S control * 100
6	HI	Norway pig herd 2	360	90	170	% of 16S control * 100
6	HI	Norway pig herd 3	1030	130	970	% of 16S control * 100
6	HI	Norway pig herd 4	1540	460	900	% of 16S control * 100
6	HI	Scottish cow	90			% of 16S control * 100
6	HI	Scottish pig herd 1	3870	1270	670	% of 16S control * 100
6	HI	Scottish pig herd 2	1610	380	580	% of 16S control * 100
6	HI	Scottish sheep	120			% of 16S control * 100
6	HI	Spanish pig herd 1	7040	1970	2460	% of 16S control * 100
6	HI	Spanish pig herd 2	6870	2050	3710	% of 16S control * 100
6	HI	Spanish piglets	7200	1210	3510	% of 16S control * 100
7	P	site 1 - pristine area			9.00E-09	copy per copy 16S
7	P	site 1 - pristine area - high flow				ug/L
7	P	site 1 - pristine area - low flow				ug/L
7	LA	site 2 - light agriculture	6.00E-07			copy per copy 16S
7	LA	site 2 - light agriculture - high flow				ug/L

Source	Disturbance Code	Station	tetW	tetQ	tetO	Units
7	LA	site 2 - light agriculture - low flow				ug/L
7	LU	site 3 - urban area	5.00E-07		2.00E-07	copy per copy 16S
7	LU	site 3 - urban area - high flow				ug/L
7	LU	site 3 - urban area - low flow				ug/L
7	HI	site 4 - heavy agriculture	6.00E-08		2.00E-07	copy per copy 16S
7	HI	site 4 - heavy agriculture - high flow				ug/L
7	HI	site 4 - heavy agriculture - low flow				ug/L
7	HI	site 5 - urban and agriculture	9.00E-07		8.00E-07	copy per copy 16S
7	HI	site 5 - urban and agriculture - high flow				ug/L
7	HI	site 5 - urban and agriculture - low flow				ug/L
8	HI	anaerobic dairy lagoon	1.00E-03		8.00E-04	copy per copy 16S
8	LA	irrigation ditch 1	1.00E-05		5.00E-06	copy per copy 16S
8	LA	irrigation ditch 10	1.00E-05		6.00E-06	copy per copy 16S
8	LA	irrigation ditch 2	1.00E-04		8.00E-06	copy per copy 16S
8	LA	irrigation ditch 3	1.00E-06		5.00E-07	copy per copy 16S
8	LA	irrigation ditch 4	1.00E-05		9.00E-07	copy per copy 16S
8	LA	irrigation ditch 5	5.00E-05		2.00E-06	copy per copy 16S
8	LA	irrigation ditch 6	3.00E-05		6.00E-06	copy per copy 16S
8	LA	irrigation ditch 7	1.00E-05			copy per copy 16S
8	LA	irrigation ditch 8	1.00E-06			copy per copy 16S
8	LA	irrigation ditch 9	9.00E-07		5.00E-07	copy per copy 16S
8	HI	microaerophillic dairy lagoon	1.00E-03		2.00E-04	copy per copy 16S

Source	Disturbance Code	Station	tetW	tetQ	tetO	Units
8	P	site 1 (aug)	6.00E-08			copy per copy 16S
8	P	site 1 (seasonal high)	4.00E-08		9.00E-08	copy per copy 16S
8	P	site 1 (seasonal low)	1.00E-07		1.00E-08	copy per copy 16S
8	LA	site 2 (aug)	1.00E-07		3.00E-07	copy per copy 16S
8	LA	site 2 (seasonal high)	6.00E-08		2.00E-07	copy per copy 16S
8	LA	site 2 (seasonal low)	4.00E-06		2.00E-06	copy per copy 16S
8	LU	site 3 (aug)	2.00E-06		9.00E-07	copy per copy 16S
8	LU	site 3 (seasonal high)	5.00E-07		1.00E-07	copy per copy 16S
8	LU	site 3 (seasonal low)	1.00E-05		4.00E-06	copy per copy 16S
8	HI	site 4 (aug)	5.00E-07		1.00E-07	copy per copy 16S
8	HI	site 4 (seasonal high)	6.00E-08		1.00E-07	copy per copy 16S
8	HI	site 4 (seasonal low)	1.00E-05		5.00E-07	copy per copy 16S
8	HI	site 5 (aug)	4.00E-07		5.00E-07	copy per copy 16S
8	HI	site 5 (seasonal high)	2.00E-07		2.00E-08	copy per copy 16S
8	HI	site 5 (seasonal low)	6.00E-06		1.00E-06	copy per copy 16S